Investigations on *Pseudomonas pseudomallei* and melioidosis

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GENERAL SUMMARY

One hundred and sixteen strains of Ps.pseudomallei collected from various countries have been examined. The morphological, cultural and biochemical properties by which the species is usually identified in the clinical laboratory have been evaluated. The tests have not excluded any of the strains examined as Ps.pseudomallei. However, the confusions that may arise in the identification of the species on the basis of 'conventional' tests have been highlighted and the need for the inclusion of some of the tests suggested by Wetmore and Gochenour (1956) has been discussed.

Colonial dissociation in Ps.pseudomallei has been shown to be a common feature. The number and frequency of such dissociants have approximated those reported for Ps.aeruginosa. A minimum period of incubation of at least 48 hours has been found to be necessary for colony differentiation. The initial recognition of Ps.pseudomallei on the basis of the rugose or ultra-rugose colony character, its 'typical' odour and lustre on plate cultures and the pellicle growth in broth, has been questioned.

Some morphological and tinctorial properties described by other workers have not been seen or have been seen or occur erratically. Electron microscopy has shown the organism to possess 2-6 flagella at one pole. The bipolar flagellation reported by some workers has not been observed. Structures resembling

fimbriae (pili) have been demonstrated.

Nine strains of the collection were examined for the presence of heat stable exotoxins.

All strains of Ps.pseudomallei have been examined for lysogenicity. The incidence of lysogeny in Ps.pseudomallei has been found to approach as much as 64% when tested against 10 selected indicator strains. That some strains are multiply lysogenic has been suspected. Mutagenic agents such as ultraviolet light and mitomycin have been found to induce the production of phage in lysogenic cultures. Bacteriocin-like inhibitors produced by a large number of these strains have interfered in the attempts to group strains on the basis of lysogenicity. A few propagated phage preparations examined in the electron microscope have shown the existence of four morphological types.

That the occurrence of bacteriocin-like inhibitions is due to more than one class of inhibitory or lytic agents including toxic alkaline metabolites and particles resembling phage components, has been considered.

Strains of Ps.pseudomallei have been shown to produce an antibacterial agent inhibitory to strains of Ps.aeruginosa and likewise some strains of Ps.aeruginosa produce agents inhibitory to Ps.pseudomallei. How these may affect 'pyocin' typing schemes in areas where melioidosis is endemic has been discussed.

Ps.pseudomallei, though lacking in the ability to produce any blue-green pigment, has shown a striking similarity to Ps.aeruginosa in inhibiting several

gram-positive and gram-negative bacteria.

Phage typing of Ps.pseudomallei strains has resulted in so many over-lapping patterns as to permit only broad groupings which, for the most part, have shown no relationship to the origin and distribution of strains or to any serological group. Much of the failure to obtain clear-cut groups could possibly be due to the phages used in the typing set.

The specificity of the propagated phages in attacking 93 of 116 strains of Ps.pseudomallei either strongly or weakly and not attacking any of the strains of Ps.aeruginosa and a number of other bacteria has once again raised the question whether a phage pool of Ps.pseudomallei could be used as an aid in differentiating the species (and if necessary, Ps.mallei) from those organisms with which it may be confused.

The fairly uniform and characteristic pattern of sensitivity of Ps.pseudomallei to antimicrobial agents used in the disc-sensitivity test in relation to those observed for Ps.aeruginosa has warranted the suggestion that it may serve as an additional aid to the identification of the species.

Since all strains of Ps.pseudomallei have been found to be serologically homogeneous in relation to agglutination tests with antisera prepared against formolised suspensions, this may offer a means of confirming the identification of the species, but the autoagglutinability of rough strains have, on occasions, been found to interfere with the conduct of such tests.

Limitations to the identification of the species by means of the agglutination test against unabsorbed sera prepared against the thermostable antigens have been shown. That strains of Ps.pseudomallei may differ in their thermostable antigenic components has been demonstrated by means of agglutinin-absorption, immuno-diffusion and immuno-electrophoresis methods. On this basis, at least 2 distinct sero-groups, one restricted to certain strains from Australia, have been found. Antigenic changes that appear to be due to phage "conversion" have been demonstrated in one instance. Changes in the degree of sensitivity to antimicrobial agents and in the production of haemolysis on blood agar that have occurred through an association with certain phages, have been reported.

FOREWORD:

The need for rapid and accurate identification of the causal organism of melioidosis in routine medical and veterinary diagnostic laboratories including those in non-endemic areas, can be foreseen from the increased number of acute and often fatal cases of the disease recorded in recent years among visitors to South East Asia. Such visitors are very likely to increase in numbers not necessarily from military operations but because of a host of other development or commercial ventures. With the increasing number of livestock importations (for breeding schemes) to enzootic areas and similar trends in the export of non-human primates and other wild or semi-wild animals from such areas, the veterinary diagnostic laboratories too may find the need to improve and intensify the methods for the bacteriological and serological diagnosis of melioidosis that is often recognised at post-mortem.

Acute melioidosis in the indigenous human and animal populations in endemic areas may seem rare but it should give no less cause for concern. Melioidosis may be a disease emerging with the changes taking place in South East Asia. These changes have been more rapid in recent years. On the one hand there has taken place extensive deforestation and more and more land has been absorbed for cultivation, particularly of paddy (rice). Unavoidable as it may be, it has led to the extension of what is considered to be the natural habitat of Ps.pseudomallei - open, wet or marshy land such as the rice 'paddies'.

There has been on the other hand a gradual build-up of a population group largely confined to a city life (because of the growth in industrial and commercial enterprises) and fast adopting a western influenced culture (food, clothing and shelter habits). Such an environment is likely to minimise the chances of acquiring a low-grade immunity through frequent exposure to mild doses of the organism as is thought to occur in population groups living in the suburban and agricultural districts. In the animal health field, the feeding, housing and sanitation systems based on western methods have gained ground mainly because of a desire to safeguard the imported breeding stock and their cross-bred progeny. This trend, most evident in piggeries, is likely to continue and lead to groups of animals or individual animals that have remained relatively unexposed to microbes such as Ps.pseudomallei. A breakdown in any one of the improved methods of rearing, which is a not infrequent occurrence in some developing nations, could lead to exposure to a number of diseases including melioidosis. The changing ecology of bacterial infections through unnecessary and improper use of antibacterial agents in therapy and in feed additives cannot be brushed aside as an unimportant factor. These are practices that are not uncommon in remote parts of the tropics. Ps.aeruginosa has become a cause for concern in most advanced countries because of its marked resistance to a wide range of modern-day antibiotics and because of the frequency with which it has been detected in recent years in clinical materials and in the hospital

environment. The sensitivity of Ps.pseudomallei to antimicrobial agents is also reported to be restricted to a few antibiotics and therefore the prospects of this organism gaining more importance as a pathogen in the tropics cannot be easily over-ruled.

The main objective in undertaking the present investigation has been to study the antigenic characters of Ps.pseudomallei that may be useful in the serological diagnosis of the disease. Since the cultures for the study were those received from various parts of the world, it was considered desirable to re-examine them for the characters defined for the species. The morphological, cultural and biochemical tests that have been widely used in diagnostic laboratories for the identification of the organism have been evaluated and some of the less well-tried methods such as bacteriophage affinities and sensitivity patterns to antimicrobial agents were also examined. In the course of the work, attempts were also made to investigate the production of antibiotic substances by Ps.pseudomallei and the possible occurrence of bacteriocinogenic strains. The assessment of these in addition to antigenic components is presented.

Part 1: Melioidosis - A general review

MELIOIDOSIS : A GENERAL REVIEW

(a) Historical.

Melioidosis is an infectious disease of man and animals caused by a Gram-negative bacillus which, though motile, has many properties in common with the organism of glanders.

Historically, the discovery of the disease dates back to the year 1912 when Whitmore and Krishnaswamy (1912) published an account of a glanders-like disease in man, in Rangoon, Burma. A year later, Whitmore (1913) reported on 38 such cases, mostly among morphine addicts, and described the morphological, cultural and pathogenic characters of the etiological agent. Since it resembled the organism of glanders in many of its characters, he proposed the name Bacillus pseudomallei for it.

Stanton and Fletcher (1921) of the Institute of Medical Research, Kuala Lumpur, Malaya, reported their encounters with the disease in laboratory rodents in 1914 and in both laboratory rodents and man in 1917. In their reports, they had noticed points of resemblance of the disease to glanders but its connection with Whitmore's bacillus was not suspected. However, by 1921, they had not only established the identity of the causative agent as B.pseudomallei but had also described new forms of the disease. Because of the varied forms of the disease, they proposed the name melioidosis derived from the name "melis" which Greek physicians had used to describe "a variety of conditions resembling glanders". They also considered

the specific name B.pseudomallei to be invalid and proposed the name Bacterium whitmori.

Since the findings of Whitmore and Krishnaswami (1912), the number of reported cases of human melioidosis, particularly in South East Asia, has gradually risen. Stanton and Fletcher (1932), in a survey of the literature then available, found that 83 cases of human infection had been reported up to that time, 39 of which had been detected at post-mortem examinations. From then onwards, the published reports of human melioidosis in Malaya have been few and far between (Green and Mankikar, 1949; Sakihara, 1952; Khaira, Young and Hart, 1959; Montgomery, 1963). However, 9 cases have been detected within a period of 3 years (1965-1969) among British and New Zealand expatriates examined at the Military Hospital, Terendak, Malaya (Thin, Brown, Stewart and Garret, 1970).

The number of cases of human melioidosis in Vietnam has far exceeded that reported for Malaya but this, to a large measure, has been due to the somewhat alarming number of cases reported recently among United States' military personnel stationed in Vietnam. In 1925, Pons and Advier (1927) diagnosed the first case of melioidosis reported from Indochina. Thereafter, many cases of melioidosis, both proven and unproven, have been reported from Indochina (Vielle, Pons and Advier, 1926; Vielle, Morin and Massias, 1926; Mesnard, Joyeux and Gaulene, 1929; Souchard and Ragiot, 1933; Roton, 1933; Couture, 1935; Long, 1937; Le Moine, Hasle and Nguyen-Duc-Khoi,

1937; Alain, St-Etienne and Delbove, 1939; Farinaud, Levy and Gros, 1950; Lajudie, Porte and Brygoo, 1952; Lajudie and Brygoo, 1953; Coudreau, 1954). Fournier and Chambon (1958) at the Pasteur Institute, Saigon, indicated that between the period 1948 to 1954, there were at least 100 cases of melioidosis among French expeditionary forces in Indochina. Thereafter, it would appear that the case reports of human melioidosis in Indochina have been presented in the main by U.S. army physicians.

Basset and Van-Van-Cua (1956), Duong-Hong-Mo and Tran-Hiep-Coung (1967), Weber (1967), Levine and Whelan (1968), Brundage, Thuss and Walden (1968) and Weber, Douglass, Brundage and Stallkamp (1969) are among those who have reported melioidosis in Vietnam. Cooper (1967a) reported on the occurrence of 35 cases with 9 fatalities during the period January through to February, 1967.

Published literature on the occurrence of human melioidosis in other parts of South East Asia is lacking, particularly since the end of the second World War and the beginning of the post-colonial era. Melioidosis has been detected in Indonesia (de Moor, Soekarnen and van der Walle, 1932; Pet and Fossen, 1934; Bezemer, 1935; van der Schaaf and Rosa, 1938; Sudibyo, 1938; Bonne, Henneman and Schijveschuurder, 1939; de Boer, 1952; Dunlop, 1952 and Brockelman, 1961); in Cambodia (Gambier, 1930); in Thailand (Peck and Zwanenburgh, 1947; Paton, Peck and Schaaf, 1947); in Singapore (Gilmour, 1931; Stanton and Fletcher, 1925b; Thin et al., 1970 and

Tarlow, 1971); in the Philippines (Gutner and Fisher, 1948) and in British North Borneo (Baird and Meers, 1965). Apart from the findings of Whitmore and Krishnaswamy (1912) and Whitmore (1913), there are but a few reports on the occurrence of human melioidosis in Burma. Knapp (1915) reported on 11 cases of 'morphine injector's septicaemia' among convicts in a Rangoon jail. This was followed by a further report by Krishnaswamy (1917). It was nearly three decades later that the next series of publications on melioidosis appeared (Cox and Arbogast, 1945; Sen, 1948; Harries, Lewis, Waring and Dowling, 1948).

Isolated cases of melioidosis have been reported in a few other tropical and sub-tropical countries. Denny and Nicholls (1927) detected a case of melioidosis in Ceylon (Sri Lanka). Mayer and Finlayson (1944) detected a case in South Africa but in this case the patient had been to the South East Asia region. Ives and Thomson (1953) reported a case in Central India. Ertug (1961) reported a case in Turkey. Cases have also been reported from the island of Guam (Mirick, Zimmerman, Manor and Humphrey, 1946), from Chad in Central Africa (Provost and Vigier, 1960) and from Ecuador (Biegeleisen, Mosquera and Cherry, 1964).

Sporadic human infections have been reported from Australia and New Guinea. Rimington (1962) reported the first six cases of human melioidosis detected in Australia. Five of these cases were also found to be suffering from other diseases. These illnesses were

thought to have lowered the patients' resistance to infection. All these cases were detected at the Townsville General Hospital, Queensland. Human melioidosis was subsequently detected in the Northern Territory of Australia (Crotty, Bromwich, Quinn and Brotherton, 1963; Bromwich and Hargrave, 1969), in Brisbane (Magee, Mitchell, Fitzwater, Christie and Rao, 1967), in Sydney (Newland, 1969) and in Thursday Island in the Torres Strait (Johnson, 1967). Kingston (1971) reported an unusual case with a prolonged latent period (24 years) in which it was thought that the infection was originally acquired during residence in New Guinea. However, the first and only report of human melioidosis in New Guinea has been made by Rowlands and Curtis (1965).

Temperate regions including both Eastern and Western Europe have had no reported cases of melioidosis except for case reports from France (Broquet, 1937; Vaucel, 1937; Alain, Saint-Etienne and Reynes, 1949; Fournier and Chambon, 1958); from England (Grant and Barwell, 1943; Maigraith and Leithead, 1964; Robinson and Ballion, 1966; Stokes and McCarthy, 1969) and from North America.

The disease position in the U.S.A. has appeared to be somewhat different from that in other parts of the Western hemisphere. An increasing number of human infections has been reported in recent years (Ziskind, Pizzolato and Buff, 1954; Prevatt and Hunt, 1957; Darby and Mendez, 1960; Rubin, Alexander and Yager, 1963; Spotnitz, 1966; Borchardt, Stanisfer and Albano, 1966; Diamond and Pastore, 1967; Baumann and Morita, 1967;

Patterson, Darling and Blumenthal, 1967; Spotnitz, Rudnitsky and Rambaud, 1967; Spotnitz, 1968; Levine and Whelan, 1968; Flemma, Di Vincenti, Dotin and Pruitt, 1969; Greenawald, Nash and Foley, 1969; Salisbury and Likos, 1970; Poe, Vassallo and Domm, 1971; Chofnas, 1972). These cases, like those that have occurred in Europe have had histories of travel or temporary residence in South East Asia or neighbouring areas and evidence points to their having contracted the disease while resident in these areas. Jackson, Moore and Sanford (1972) reported that from April, 1965 to December, 1969, there had been 187 cases of melioidosis with 13 deaths among United States Army personnel who were in or had been in Vietnam.

Apart from melioidosis reported in United States citizens returning from South East Asia, there are also a few reports of melioidosis in United States citizens who had returned after residence in the Panama Canal Zone (Joy, Scalletar and Sodee, 1960; Rubin, Alexander and Yager, 1963). Cases have also been reported in persons who have never left the United States of America (McDowall and Varney, 1947; Beamer, Varney, Brown, McDowall and Eck, 1948; Garry and Koch, 1951; Osteraas, Hardman, Baas and Wilson, 1971). Green and Tuffnell (1968) reported a case in Canada in which the patient, a laboratory research worker, had acquired the infection when handling a culture of Ps.pseudomallei.

Veterinary interest in this disease lies in the fact that the organism has been recovered from laboratory and

domestic animals and occasionally from captive wild animals. Such isolations have been made from carcases showing a variety of lesions including septicaemia, localised or generalised pneumonia and pyaemia with multiple caseous nodules or abscesses of the visceral organs - generally the lungs, spleen, liver, kidney, lymph nodes and infrequently the pancreas and the adrenals. In male subjects, miliary abscesses have sometimes been observed in the testes and epididymis. Cases have also been reported where the organism had been isolated from the urine, uterine and nasal discharges, brain and cerebrospinal fluid, abscesses in the joints of the limbs, intestines and from an aborted goat foetus and a pig foetus.

Stanton and Fletcher (1925a) reported their encounters with the naturally occurring disease in their laboratory animal colonies (rabbits and guinea-pigs) at the Institute of Medical Research, Kuala Lumpur, Malaya in 1913 and 1917. Every case had ended fatally and the infection had persisted for nearly 11 years. They attributed the infection to the consumption of infected foodstuffs which they thought had been contaminated with the excreta of wild rats carrying the infection. This assumption was based on the fact that they had examined several wild rats (Mus griseiventer, Bonhote) some of which had died from the disease contracted under natural conditions. Stanton and Fletcher (1921 and 1925a) also reported their observations of naturally occurring melioidosis in a domestic cat which was said to have died

of a "haemorrhagic distemper". Again, in 1927, Stanton, Fletcher and Symonds (1927) isolated the organism from a transient nasal discharge in a horse in Malaya which had been imported from Australia about 6 months earlier. The animal was destroyed 18 months later but at post-mortem examination there was no evidence of any lesions suggestive of melioidosis nor was the organism isolated. In a subsequent report, Stanton and Fletcher (1932) mentioned the isolation of B.whitmori from the nodules in the spleen and lung of a dog in Malaya which had died of a "distemper-like" illness. The next published report of melioidosis in Malaya appeared nearly 20 years later when Davie and Wells (1952) published their findings on equine melioidosis. They reported the occurrence of 3 fatal cases of the disease in race horses during the period 1947 to 1949. These animals were imported into the country, two from Australia and one from the United Kingdom. In none of these was the diagnosis established until after death. According to Retnasabapathy (1959), melioidosis in goats was recognised in Malaya in 1953 when an outbreak occurred at the Agricultural Experimental Station, Serdang. Pfeifferella whitmori had been isolated from various organs sent to the Veterinary Research Institute, Ipoh, Malaya. The first two cases of porcine melioidosis were detected in Malaya in 1956 when Retnasabapathy (1959) isolated Pf.whitmori from urine collected at the post-mortem examination of a Middle White sow. Omar (1963) published the histopathological findings on 78 cases of

melioidosis which had been diagnosed at the Veterinary Research Institute, Ipoh, within the 4-year period from 1958-1961. The materials included those from 37 culturally positive cases in goats detected since 1958 within the Federation of Malaya and Bornean territories; 40 cases of melioidosis in pigs detected in Malaya in 1961 and a single case of melioidosis in a horse detected in Malaya in 1960. This study included specimens from a case of melioidosis in a wild goat (Capricornis sumatrensis) described by Lee (1961) and from an outbreak of melioidosis in a herd of imported European breeds of pigs reported by Omar, Cheah and Mahendranathan, 1962. Since then, although a wide host range of the causative organism has been reported, little has been said on the incidence and the economic importance of the disease in domestic animals in Malaya. The organism has been isolated from a goat-foetus (Retnasabapathy, 1966), a macaque monkey (Macaca sp.) (Retnasabapathy and Joseph, 1966), a calf (Chooi, Lim, Shanmugarajah and Rajagobal, 1967), a parrot (Amazoa albifrous) (Lim and Tan, 1967), a sheep (Lim and Retnasabapathy, 1967), a spider-monkey (Brachytellis arachnoides) (Lim and Mukundhan, 1968), a gibbon (Hylobates lar.) (Lim and Mukundhan, 1968), an orang-utan (Fong and Lim, 1969), a macaque (Macaca nemestrina) (Strauss, Jason, Lee and Gan, 1969) and a buffalo calf (Omar, Mohd. Anwar and Joseph, 1970). The FAO/OIE/WHO Animal Health Yearbooks (1971 and 1972) indicate that melioidosis in goats, pigs and horses continues to occur sporadically, in West Malaysia

(former Federation of Malaya).

Animal Health Yearbooks (1962-1970) also show that Sarawak and Sabah (former British North Borneo) have had a low but undetermined incidence of melioidosis in livestock. It has been reported that melioidosis occurs in goats in Sarawak and in caprine, ovine and porcine populations in Sabah. A neighbouring territory, the Sultanate of Brunei, has reported melioidosis in goats in recent years (Yearbook, 1968).

Published literature on melioidosis in domestic animals in other parts of South East Asia where human melioidosis has been detected appear to be negligible. Nguyen-Ba-Luong (1956) described an epizootic of melioidosis in swine in Vietnam. Phung-Van-Dun (1957) has observed melioidosis in buffaloes, cats and dogs. Nguyen-Van-Ai (1960) has reported on the occurrence of sporadic cases in pigs in Vietnam and Stedham (1971) reported melioidosis in 4 German-shepherd dogs belonging to the United States Army in Vietnam. Thonn, Lebon, Saphon and Triau (1960) reported on outbreaks of melioidosis in swine and horses in Cambodia. From the FAO/OIE/WHO Yearbooks (1962-1970), it would appear that sporadic infections have continued to occur in pigs.

Apart from the earlier documentation of equine melioidosis in Indonesia (de Moor et al., 1932 and de Boer, 1952) and in the Philippines (van der Schaaf and Rosa, 1938), there is little information on the occurrence of melioidosis in animals in Indonesia, Philippines, Thailand, Laos and Burma. The data in the FAO/OIE/WHO

Animal Health Yearbooks (1962-1970) indicate that melioidosis occurs in pigs and horses in Burma and in horses alone in Indonesia. Laos, Philippines and Thailand are said to be free. The identification of Ps.pseudomallei from a "glandered horse" in the Philippines (Rubin et al., 1963) and the serological evidence indicative of subclinical or unrecognised mild melioidosis in certain native population groups in Thailand (Nigg, 1963) leave room to speculate on the occurrence of melioidosis in these areas.

Melioidosis has also been detected in other widely scattered tropical islands or regions. Nicholls (1930) isolated B.whitmori from a splenic abscess in a cow in Ceylon which had died following rupture of the abscess. Girard (1936) isolated Malleomyces pseudomallei from the submaxillary lymph nodes of a pig in Madagascar. Sutmoller, Kraneveld and van der Schaaf (1957) reported abscesses in the lymph nodes of sheep, goats and pigs at the slaughterhouse in Aruba in the Dutch Antilles. They also mentioned an outbreak in which 25 sheep in a flock of 90 had died within a few weeks and those that survived had become thin and developed a polyarthritis. Necropsies had revealed abscesses in the lungs, liver, spleen and the kidneys. Organisms were isolated from these animals and were shown to correspond to the morphological, cultural, biochemical and antigenic characters of M.pseudomallei. All strains of the organism tested proved to be pathogenic on inoculation into rabbits and mice. The FAO/OIE/WHO Animal Health

Yearbooks (1962-1970) state that melioidosis occurs in pigs in Madagascar. The same Yearbooks indicate that during that period, the disease has not been observed in Ceylon. There has been no mention of the Dutch Antilles and its disease position.

Melioidosis has also occurred in a goat in Chad (in Central Africa) (Provost and Vigier, 1960) and in a horse in the Suez Canal zone (MacLennan, 1953). Baharsefat and Amjadi (1970) reported on the detection of equine melioidosis in Iran. The occurrence of melioidosis in goats in Chad and in goats and sheep in Iran has been reported in many of the FAO/OIE/WHO Animal Health Yearbooks (1962-1970). Whether by an error or not, it has also been reported that melioidosis has occurred in goats, sheep, pigs and horses in the Bahamas (FAO/OIE/WHO Animal Health Yearbook 1967 and several others in the series 1962-1970).

In recent years, a number of cases of melioidosis in domestic animals and some wild animals held in captivity have been reported from parts of Australia and her neighbouring territory of Papua and New Guinea. The occurrence of melioidosis in domestic animals in Australia can be traced back to the year 1949 when Cottew (1950) and Cottew, Sutherland and Meehan (1952) isolated M.pseudomallei from fatal infections in sheep in North-western Queensland. Subsequently, Lewis and Olds (1952) and Olds and Lewis (1954) reported on the occurrence of the disease in goats in the Townsville area in Queensland. Again, Olds and Lewis (1955) isolated M. pseudomallei from

a fatal case of acute melioidosis in a pig in Townsville on one of the farms where the disease had previously been detected in goats. M.pseudomallei was isolated from the lungs, liver, spleen, lymph nodes and the tonsils. In 1963, Laws and Hall reported on cases of melioidosis encountered in domestic animals in parts of North Queensland and in particular, the Townsville area, during the period June, 1956 to July, 1961. These workers recovered Pseudomonas pseudomallei from 115 naturally infected animals which included 75 pigs, 23 sheep, 14 goats, 3 cattle and a horse. Of these animals, 104 had shown no symptoms and the majority of the isolations had been from abscesses in the lymph nodes, lungs or spleen, detected during meat inspection at slaughter-houses. Four of the 12 animals (2 cows, 1 goat and 1 horse) that had clinical evidence of the disease showed infections of the central nervous system. The Annual Reports of the Department of Agriculture and Stock, Queensland, Australia (1960/61 and 1961/62) state that melioidosis abscesses had been seen in a number of sheep and pigs and that 15 strains in all were recovered from these animals and from swamp water. The infections in sheep in Australia were thought to be due to grazing on pastures where the disease had occurred previously. Since then, it would appear (FAO/OIE/WHO Yearbooks 1963-1970) that melioidosis though confined to certain regions of Australia, has occurred sporadically in goats, horses, sheep and pigs. Three other cases of melioidosis reported from Australia during this period are of special interest.

Tammemagi and Johnston (1963) isolated Ps.pseudomallei from the lungs, liver, spleen and kidney of an orang-utan (Simia satyrus) at a zoo in Townsville, Queensland. This animal had been imported from North Borneo (Sabah, Malaysia) four years previously. Ketterer and Bamford (1967) reported on the detection of melioidosis in lambs from two adjoining farms in the Chittering Valley near Perth in South Western Australia (an area lying between 31° and 32° South). Rogers and Andersen (1970) reported on the isolation of Ps.pseudomallei from the liver, kidneys, lung and stomach contents of an aborted piglet. The sow was slaughtered one month after the abortion and Ps.pseudomallei was recovered from abscesses found in the spleen, liver, kidneys and mesenteric lymph nodes.

As in Australia, the disease in Papua and New Guinea was first recognised in animals. Egerton (1963) isolated Ps.pseudomallei from a tree-climbing kangaroo (Dendrolagus sp.) captured from a remote area North-east of Port Moresby in Papua and held in captivity at the Port Moresby Zoo for nearly two years. Clinically, the case was characterised by posterior paralysis. At necropsy, the organism was isolated from the lumbar region of the spinal cord and from abscesses in the liver and spleen. A year later, Egerton (1964) isolated Ps.pseudomallei from a case of bovine melioidosis and Rampling (1964) recorded the isolation of Ps.pseudomallei from abscesses in the spleen of a four-months old pig from the same farm as Egerton's case. A low sporadic occurrence of melioidosis

in pigs in Papua and New Guinea is indicated in the FAO/OIE/WHO Yearbooks 1968-1970.

Melioidosis in domestic or wild animal species has never been recorded in Europe but the occurrence of glanders in man and the Equidae has been well recognised since early times. Published reports of glanders include an article by Bernstein and Carling (1909) who noted that their strains of glanders bacilli from six classical cases of human glanders in Great Britain, were motile. Their identifications were made some time before the discovery of the causal organism of melioidosis. However, it is perhaps pertinent to ask whether similar errors could not have been made in veterinary circles in Europe, in their campaigns to eradicate equine glanders. Contrary to the findings of Stanton and Fletcher (1932), Bozzelli (1930) and MacLennan (1953) found that horses affected with melioidosis gave positive reactions to the mallein test. Cravitz and Miller (1950a) have reported that the complement-fixation test does not differentiate between Malleomyces mallei and M.pseudomallei.

Melioidosis in domestic animals has not been recorded in the two American continents but with the increase in importation of non-human primates into the U.S.A., there have been reports in very recent years, of the occurrence of melioidosis in such primates. According to the Weekly Morbidity and Mortality Report (10th May, 1969, p.163-164) of the National Communicable Disease Centre, Atlanta, Georgia, melioidosis was detected in a stump-

tailed monkey (Macaca speciosa) which had been imported from Thailand. Subsequently, Kaufmann, Alexander, Allen, Cronin, Dillingham, Douglas and Moore (1970) recorded three separate outbreaks of melioidosis in imported non-human primates and one of these outbreaks probably included the case of melioidosis reported by Butler, Schmidt and Wiley (1971). Some of the animals involved in these outbreaks had been imports from different geographical regions (South East Asia, India and Africa) but had been in the premises in the U.S.A. for periods of time ranging from six months up to three years. It would suggest a long incubation period of the disease in some of these animals.

(b) Distribution and species of animals affected.

Two striking features of melioidosis are its comparatively limited geographic distribution and the wide variety of animal species affected. Most countries where melioidosis has occurred are in the tropical and sub-tropical belts - Vietnam, Cambodia, Laos, Thailand, Burma, Phillipines, Sabah, Sarawak, Brunei, Malaya, Singapore, Indonesia, Ceylon, New Guinea, parts of Australia, Central India, Madagascar, Suez Canal zone, Turkey, Chad in Central Africa, Panama Canal zone Ecuador and Guam. Although in recent years cases have been reported outwith 20°N and 20°S (Turkey, Central India, Chittering Valley near Perth in Australia), the suggestion by Redfearn, Palleroni and Stanier (1966) that areas endemic for melioidosis are to be found

within this zone seems a reasonable one. The cases reported from other areas (temperate regions) have been those of patients who had apparently contracted the disease during visits to endemic areas. The sources of infection in the few patients who had apparently never left the U.S.A. were not found and therefore this aspect must remain unexplained.

Thus man, almost all domestic animals (horses, cattle, sheep, pigs, goats, dog and cat) and some wild animals (apes, marsupials, a wild goat and a parrot) held in captivity at zoos or laboratories, have succumbed to the infection. Outbreaks affecting laboratory rodents (guinea-pigs, mice and rabbits) have been recorded. The wide host range is suggestive of a lack of host specificity on the part of the organism.

(c) Source of infection and the reservoirs.

For many decades, melioidosis has been regarded as a disease primarily of rodents, the organism being perpetuated by "carriers", principally wild rats. Dissemination of the disease was thought to be due to the consumption of food and water contaminated with the excreta of such carriers. The occasional infections in man and domestic animals were thought to be due probably to environmental factors that may permit the entry of the organism from such contaminated materials. These views have stemmed mainly from the publications of Stanton and Fletcher (1925a). As a result, the disease has come to be regarded as a zoonotic problem.

Girard (1936) however, reporting melioidosis in a pig in Madagascar, remarked that he found no evidence of the disease in rats although he had, over a period of 15 years, inoculated materials from rats into guinea-pigs to survey for plague. Delbove and Reynes (1942), in a more extensive survey of the rat population in Vietnam found only one infected rat in the 20,000 examined. Harries et al. (1948) examined more than 500 rats in Rangoon, Burma, but found no evidence of the disease. Brockelmann (1961) examined 935 rats in the course of surveys for plague in Java and he too found no evidence of melioidosis. Strauss, Ellison, Gan, Jason, Macarelli and Rapmund (1969) in an extensive survey covering many thousands of rats in Malaya were unable to isolate Ps.pseudomallei from any rats. These findings have helped to dispel the belief that rats are the principal source of infection.

The ground and stagnant waters in endemic areas have been considered as principal reservoirs for the organism, man and animals acquiring the infection directly from such sources. Some of the earlier cases on record (Ragiot and Delbove cited by Huard and Long, 1937; Marque and Raynal, 1935; Le Moine, Hasle and Nguyen-Duc-Khoi, 1937) have indicated that fatal melioidosis occurred within 8 to 24 days of automobile accidents during which the victims had sustained lacerations which had been contaminated with muddy water. Chambon (1955) isolated M.pseudomallei from 5 out of 150 samples of mud and water from stagnant pools, swamps

and rice fields in Vietnam. Laws and Hall (1964) recovered Ps.pseudomallei on two occasions from swamp waters in farms in North Queensland where melioidosis had been detected previously in animals. Ellison, Baker and Mariappan (1969) isolated Ps.pseudomallei from the Gambok river near Kuala Lumpur, Malaya. Strauss, Jason and Mariappan (1967) and Strauss, Groves, Mariappan and Ellison (1969) described their findings on the distribution of Ps.pseudomallei in soil and surface water in Malaysia. Strauss et al (1969) also indicated a high recovery rate of the organism from rice fields and other open lands but a low recovery rate from the soils and surface waters of forested lands. Miller, Pannell, Cravitz, Tanner and Ingalls (1948) showed that the organism could survive in tap water for several weeks. Mixed with dry garden earth and kept in a dessicator at 26°C., the organism had remained infective for guinea-pigs for 27 days. Fletcher (1929) also reported that the organism can survive in dust, dirty water and faeces for 4 weeks, in urine for 2 weeks and in putrid cadavers for 1 week. Leclerc and Sureau (1956) isolated bacteriophages from stagnant waters in Hanoi, North Vietnam, which specifically attacked strains of M.pseudomallei in their collection. In describing outbreaks of melioidosis in swine (Nguyen-Ba-Luong, 1961) and in guinea-pigs (Joubert and Phung Van Dam, 1958) it has been stated by these workers that the organism was also isolated from feed and water derived from environmental sources.

Such findings, either alone or taken in conjunction

with the evidence of the occurrence of sub-clinical, latent or inapparent melioidosis infections in man and of latent infections in domestic animals (Girard, 1936), may explain how the causative organism may survive in nature. The recrudescence of latent infections into clinical forms of the disease (Fournier and Chambon, 1958; Prevatt and Hunt, 1957), the shedding of the organism in the discharges of infected animals and man (Stanton and Fletcher, 1927; Souchard and Ragiot, 1933; Long, 1937; Olds and Lewis, 1954; Retnasabapathy, 1959; Laws and Hall, 1964 and Rogers and Anderson, 1970), the ability of the organism to survive in faeces (27 days), urine (17 days) decomposing carcases (8 days) and its considerable resistance to drying (Fletcher, 1929) coupled with the comparatively low levels of human and animal sanitation in the developing regions may assist in concentrating the organism and polluting the inhabited, open, marshy lowland pockets in these regions.

The evidence for the occurrence of sub-clinical melioidosis has come from many sources. Nigg and Johnston (1961) observed what appeared to be sub-clinical melioidosis in experimentally infected monkeys and guinea-pigs. Dannenberg and Scott (1958a,b) showed an acquired immunity in mice by experimental inoculation of live virulent or avirulent organisms. Brygoo (1953a), in Saigon, Vietnam, detected agglutinins to Pf.whitmori in 465 sera submitted for routine laboratory examinations. In the sera from non-febrile patients (Wassermann sera)

the agglutinin titres were not found to exceed a 1:20 dilution but sera from patients suspected of enteric fever had often showed titres to the Pf.whitmori antigen of 1:40 or over. Asians were found to have agglutinins more often than Europeans. Spotnitz (1967) found that 1.1% of 372 unselected United States soldiers from Vietnam, hospitalised in the U.S.A. had significant antibody titres to the melioidosis haemagglutination test without any clinical evidence of the disease. In recent years, two more serologic surveys have been carried out on United States military personnel who had returned from Vietnam. Kishimoto, Brown, Blair and Wenkheimer (1971), using the haemagglutination test (polysaccharide antigen), examined single serum samples each from 400 military personnel from Vietnam and 100 samples from military personnel who had not been to Vietnam. They found that 27 (6.75%) of the personnel from Vietnam and 2 (2%) of those in the control group had antibody titres ranging from 1:10 to 1:40 and that 7 (1.7%) of those from Vietnam and 1 in the control group had antibody titres of 1:80 or over.

Clayton, Lissella and Martin (1973), using the haemagglutination test procedure of Alexander, Huxsoll, Warner, Shepler and Dorsey (1970), examined serum samples from 412 military personnel returning from Vietnam and 606 serum samples from a control group of personnel who had not been to Vietnam. They found that 11.8% of the test group and 2.8% of the control group had titres

of 1:20 or less and that 8.9% of the test group and 2.9% of the control group had titres of 1:40 or more.

Nigg (1963) conducted a serological survey using both complement fixation (CF) and the haemagglutination (HA) tests on sera from healthy military volunteers in Thailand and on sera from a control group based in the U.S.A. She found 8.3% of 337 healthy Thai group males and 1 of Thai group females positive to the CF test with none positive in the control group of 138 personnel. She also found that, of the 405 sera in the Thai group, 118 (29.1%) were positive for the haemagglutination test using the polysaccharide antigen, with titres ranging from 1:5 to 1:10,240 and, of the 132 sera in the control group, 9 (7.4% sic) were positive with titres ranging from 1:5 to 1:120, although only 2 of these had titres of 1:40 or over. Moreover, 8 of these 9 positive sera were from navy personnel whose previous history concerning visits to endemic areas was not known

Strauss, Alexander, Rapmund, Gan and Dorsey (1969), using a modification of the indirect haemagglutination test of Ileri (1965), examined 1592 serum samples from normal human population groups (forest-dwelling aborigines; Army recruits from rice-growing States, Army recruits from other States; Army engineers; military camp ground keepers; oil-palm and rubber estate workers, mostly labourers) in Malaysia and 200 serum samples from a control group resident in the U.S.A. Only 7 (3.5%) of the control sera were found

to have titres of 1:10 or 1:20 but 235 (14.1%) sera from Malaysia showed titres from 1:10 to 1:2,560. On the basis of their findings in the control group sera, Strauss et al. considered a titre of 1:40 to be specific for melioidosis haemagglutinating antibody. Thus 116 (7.3%) in the test group were considered positive for the HA test. Their findings also suggested that melioidosis occurs more often in those whose occupation involves prolonged exposure to water containing the organism, such as rice farmers.

The methods applied by these different workers are not strictly comparable and there appears to be no general agreement on what constitutes an acceptable melioidosis haemagglutination titre. Thus, Malizia, West, Brundage and Walden (1969) considered a titre of 1:80 to be significant, while Strauss et al. (1969) regarded 1:40 as a significant titre and Khaira et al. (1959) noted that cases with overt melioidosis gave titres no more than 1:40. Nigg's finding of 30% positive cases to the polysaccharide HA test included reactions at a serum dilution of 1:5. In assessing these results some workers have made no allowance for non-specific haemagglutination or agglutination. Whilst allowing for such discrepancies, it still becomes apparent that a considerable proportion of the 'reactors' in each study carries a high antibody titre. Nigg and Johnston (1961) demonstrated by animal experiments that serological titres do not remain elevated indefinitely after infection and Alexander et al. (1970) found that

only a small number of human melioidosis cases showed detectable antibody titres up to 2 to 3½ years and only one case showed detectable antibody titres up to 7 years. A significant antibody titre may reflect a past infection and in the absence of a history of recent illness, the findings in the serological surveys of the healthy test groups mentioned above are indicative of the occurrence of unrecognised or asymptomatic melioidosis either in the form of a low-grade, inapparent infection and subsequent recovery of a latent localised infection persisting without a clinical breakdown. There appear to be no serological surveys conducted in animal populations except for one by Cook (1962) who used the CF test and found only isolated cases in man and animals showing any significant titres.

(d) Transmission.

The route of infection has remained uncertain. Oral, dermal and respiratory routes have been suggested (Stanton and Fletcher, 1925; Chambon, 1955; Fournier and Chambon, 1958; Duroux, 1965; Sheehy et al., 1967; Green and Tuffnell, 1968; Greenawald et al., 1969; Thin et al., 1970). Experimentally, infection has been produced in animals by the oral route (Miller, Pannell, Cravitz, Tanner and Rosebury, 1948); the respiratory route (Rosebury, 1947; Dannenberg and Scott, 1958a) and by skin wounds by scarifying the skin and applying the organisms (Stanton and Fletcher, 1932; Vaucel, 1937). Blanc and Baltazard (1941 & 1942) were able

to transmit the infection to guinea-pigs by the bites of the rat flea (Xenopsylla cheopis) and the mosquito (Aedes aegypti).

The association of lung abscesses with pyaemia in human melioidosis has supported the suggestion of the respiratory tract as a portal of entry (Thin et al. 1970). However, the cutaneous route has been favoured by a number of other workers (Fournier and Chambon, 1958; Duong-Hong-Mo and Tran-Hiep-Cuong, 1967). Many of the cases recorded by Whitmore and Krishnaswamy (1912), Whitmore (1913a) and Knapp (1915) were in morphine injectors. Three other fatal cases of human melioidosis reported in earlier literature (Ragiot and Delbove, 1937; Marque and Raynal, 1935; Le Moine, Hasle and Nguyen-Duc-Khoi, 1937) had apparently contracted the disease through lacerations sustained in automobile accidents, which had been contaminated with mud. A high incidence of melioidosis was also observed in French and U.S. infantrymen who had sustained burns, lacerations or other open wounds during military operations in Vietnam (Fournier, 1960; Rubin et al., 1963; Flemma et al., 1969). Nearly three-quarters of 113 cases of melioidosis diagnosed in United States soliders in Vietnam within a period of 13 months were from infantry divisions (Piggott and Hochholzer, 1970). Kishimoto et al. (1971) showed a higher percentage of 'reactors' to the haemagglutination test in wounded United States soldiers from Vietnam in comparison with soliders in other test groups. This pattern is comparable with that observed in a serological

survey in population groups in Malaysia. The survey in Malaysia (Strauss et al., 1969) revealed that antibodies against Ps.pseudomallei were most frequent in those whose occupation involved prolonged exposure to waters containing the causative organism. These findings suggested cuts and abrasions on the extremities as a probable portal of entry.

Contact infections from man to man, animal to animal or animal to man have not been observed.

(e) Clinical disease.

The incubation period of the disease in man and domestic animals has not been defined but experiments in laboratory animals show that it can vary from a day to several weeks.

Several factors appear to predispose man and animals to infection leading to overt disease. Many of the cases observed in man have been among troops exposed to climatic and military stresses. Outbreaks or isolated cases of acute melioidosis have been frequent in imported breeds of domestic animals or their cross-bred progeny. Melioidosis was recorded in imported horses (Stanton et al., 1927; Davie and Wells, 1952), in imported pigs and their cross breds (Nguyen-Ba-Luong, 1956; Retnasabapathy, 1959; Thonn et al., 1962; Omar et al., 1962 and Strauss et al., 1967) and in imported dogs (Moe et al., 1972 and Stedham, 1971). Debilitating diseases appear to have predisposed human beings or animals to the illness, although a number of

cases have also been reported in previously healthy subjects. The majority of human cases recorded in Burma (Whitmore, 1913a; Knapp, 1915) were among debilitated persons many of whom were morphine addicts. Three of the six initial cases recorded in Australia (Rimington, 1962) were in diabetics and two others had chronic systemic derangements (chronic nephritis in 1 case and cystic disease of the lungs associated with pregnancy in the other case). Diabetes mellitus was also found in cases reported by other investigators (Alain et al., 1949; Crotty et al., 1963; Robinson and Ballion, 1966; Magee et al., 1967; Thin et al., 1970; Jackson et al., 1972 and Chofnas, 1972). Two of the 10 human cases described by Thin et al. (1970) also had cirrhosis of the liver. The cases reported by Tarlow (1971) had a chronic granulomatosis. Concomitant bacterial or viral infections also have been observed in cases of melioidosis (Grant and Barwell, 1943; Alain et al., 1949; Fournier and Chambon, 1958). Surgical operations also appear to have activated the disease (Roques and Dauphin, 1943; Spotnitz, Rudnitzsky and Rambaud, 1967).

The overt disease in human beings may manifest itself in several forms. Many attempts have been made to classify these forms and one such was that of Alain, Saint Etienne and Reynes (1949) but the symptoms have often overlapped from one form to another and are in themselves protean. The commonest form of the disease to be recognised by the earliest investigators (Whitmore,

1913; Knapp, 1915; Stanton and Fletcher, 1925band Biegeleisen et al., 1964) was the acute condition. It was marked by the sudden onset of the symptoms with chills, fever and prostration followed by a gastro-enteritis, pneumonitis or a septicaemia with death supervening in two to four days. However, the majority of cases reported since the second World War have been of the sub-acute form which runs a clinical course of one to several weeks (Sakihara, 1952; Khaira et al., 1959; Rimington, 1962, Crotty et al., 1963; Patterson et al., 1967; Duong-Hong-Mo, 1967; Spotnitz et al., 1967; Baumann et al., 1967; Diamond et al., 1967; Brundage et al., 1968; Weber et al., 1969 and Thin et al., 1970). In such cases, the primary infection has been a pneumonitis followed by septicaemia and widespread abscess formation or it may occur initially as a skin or subcutaneous lesion with lymphangitis or lymphadenitis followed by septicaemia and general organ involvement. This form of the disease has been described as a chronic condition and is thought to develop in patients who survive the acute or sub-acute infection, although chronic forms without such antecedent infections have been reported. The course of chronic infections have been reported to run from several weeks to several years. Prevatt and Hunt (1957) in reporting a case of chronic melioidosis found only 17 such cases previously recorded and considered the chronic form to be a rare manifestation but since then a few more cases have been reported (Darby and Mendez, 1960; Borschardt et al.,

1966; Maigraith and Leithead, 1964; Magee et al., 1967; Levine and Whelan, 1968; Newland, 1969 and Kingston, 1971). Five human cases reported by McDowell and Varney (1947), Prevatt and Hunt (1957), Guillermand et al., (1964), Newland (1969) and Kingston (1971) respectively, and three of the five cases in non-human primates reported by Kauffmann et al. (1970) are interesting because these suggest latent infections of many years duration. If Kingston's (1971) case contracted melioidosis in New Guinea some 24 years earlier, then the prolonged course of the disease in this patient becomes the longest on record followed closely by that of Newland's (1969) and that of McDowell and Varney's (1947) which were thought to have had latent infection for 18 and 17 years, respectively.

Apart from these forms of the disease, comment may be made on two other forms which have been reported. Green and Mankikar (1949), Sudibyo (1938), Hasle and Nguyen-Duc-Khoi (1937) reported cases of chronic afebrile melioidosis with lesions confined to the skin. Pustular rashes and bullae (abscesses in the subcutaneous tissues and muscle) have been observed in later stages of septicaemic infection in human beings (Stanton and Fletcher, 1925b). Diamond and Pastore (1967) reported a case of septic arthritis in a human being.

Acute and sub-acute melioidosis in domestic animals has been reported (Davie and Wells, 1952; Cottew et al., 1952; Thonn et al., 1960; Omar et al., 1962; Omar, 1963; Stedman, 1971; Nguyen-Ba-Luong, 1961) but

chronic infections, generally recognised at necropsy, appear to have been the commonest (Nicholls, 1930; Girrard, 1936; MacLennan, 1953; Olds and Lewis, 1954; Sutmoller et al., 1957; Provost and Vigier, 1960; Laws and Hall, 1963). This may not be the true picture however, as the known enzootic areas, under-developed and geographically remote may be poorly provided with veterinary personnel both in the field as well as in the laboratory. Regular visits for the diagnosis of disease and continued therapy of the sick animal becomes almost impossible except in Government-owned farms that are generally situated in areas easily accessible to regional veterinarians. Thus the tendency to resort to a single dose (or to doses at irregular intervals) of a wide-spectrum and reportedly long-acting antibiotic (or a combination of antibiotics) at the first sign of any illness accompanied by an elevated body temperature with no follow-up therapy thereafter is a not infrequent procedure in these areas. Such therapeutic regimens may lead to the death of the animal without evidence as to its cause or it may mask the infection or alter the course of an acute infection. Reports on acute human melioidosis indicate that the wrong choice of antibiotics and the improper dosage regimens with right antibiotics have often led to the death of the patient or to the chronic form of the infection.

(f) Pathology.

Studies on the pathological and histopathological features of melioidosis in humans (Gutner and Fisher, 1948; Greenawald et al., 1969; Foley, Greenawald, Nash and Pruitt, 1969; Piggot and Hochholzer, 1970), domestic animals (Omar, 1963) and in laboratory rodents (Dannenberg and Scott, 1958a) indicate that lesions are similar among the different species but are not pathognomonic of the disease. The lesions may vary according to the form and duration of the illness. In general, it may be said that lesions of melioidosis range from suppurative abscesses to caseous granulomata (Borschardt et al., 1966; Gutner and Fisher, 1948; Mirick et al., 1946; Patterson et al., 1967 and Spotnitz et al., 1967). These may be generalised affecting many organs or may be localised to a single organ. Some of these variable clinical and pathological manifestations may be due to factors such as the virulence and initial dose of the infecting strain and the resistance of the affected host. Both virulent and avirulent strains are known to occur naturally for they have been isolated from the soil and swamp waters in some endemic areas using selective culture media (Fournier and Chambon, 1958). Experimental infections in mice have shown that large doses of virulent strains lead to acute infections and smaller doses to chronic infections (Dannenberg and Scott, 1958a). Experimental studies in mice have also shown that a degree of immunity could be conferred by inoculating a

large dose of a live avirulent strain of the organism or by a small dose of a virulent strain (Dannenberg and Scott, 1958a,b). Therefore, leaving aside the aspect of non-specific resistance, it would be possible for a subject in an endemic area to acquire a degree of resistance through exposure to suitable doses of either virulent or avirulent strains found free-living in the environment. A low level of such acquired immunity may not prevent a subject from succumbing to a subsequent virulent infection but it may alter its course and thereby its clinical and pathological manifestations.

A knowledge of the pathogenesis of the disease would undoubtedly help in further elucidating the clinical and pathological pictures of the disease but our understanding has been meagre. Our knowledge has been largely derived from observations in the pathogenesis of melioidosis in experimentally infected laboratory rodents. Dannenberg and Scott (1958a,b) observed that the forms of the disease and the lesions in mice may resemble those in man. They also indicated that the toxins which the organism may produce in vivo may be a significant factor in the causation of the observed lesion. Evidence that the toxins may be produced in vivo has been based on the similarity of the response and the pathology in infected mice and in mice inoculated with bacteria-free culture filtrates.

The production of toxin was first shown by Legroux, Kemal-Djemil and Jeremac (1932). Subsequently, Nigg

Heckly and Colling (1955) demonstrated a lethal toxin in sterile culture filtrates of M.pseudomallei by inoculating mice and hamsters intra-peritoneally. Mice were found to be sensitive, many of them dying within 24 hours of the injection without showing any gross or histopathological lesions. Those that survived a few days had shown profuse lachrymation and paralysis of the hind legs. Nigg et al. (1955) also showed that intra-dermal inoculation of such culture filtrates into guinea-pigs produced, within minutes to hours, an oedematous, erythematous, haemorrhagic lesion which often progressed into a necrotic lesion with surrounding oedema before healing. Liu (1957) in demonstrating the production of haemolysins of Pseudomonas spp. confirmed these observations. Later studies by Heckly and Nigg (1958) showed that at least two different thermolabile exotoxins, a lethal toxin and a necrotoxin, were responsible for the two different reactions. They found no evidence of a haemolysin in culture filtrates but found a pronounced proteolytic activity suggestive of an association with an enzyme or enzymes. Heckly (1964) extended these studies to show that an alcohol-precipitated fraction of culture-filtrates not only killed mice when injected intra-peritoneally and produced a dermonecrosis in guinea-pigs when injected intra-dermally, but also exhibited proteolytic and anti-coagulant activities. By treatment of crude toxin preparations with antisera prepared against acid- or alkali- treated fractions, he was able

to show that the necrotoxin could be precipitated with the proteolytic activity, leaving the lethal toxin (with anti-coagulant activity) in the supernatant fluid. All attempts to separate the necrotoxin from the proteolytic activity failed and it was therefore considered that the necrotoxicity was a function of the enzymatic activity. The mechanism of the action of the lethal toxin has remained obscure.

Apart from the exotoxins, an active endotoxin similar to the classical endotoxin of Gram-negative bacteria has been demonstrated. Moreover, it has been shown (Rapaport, Miller and Ruch, 1961) to be capable of eliciting a generalised Schwartzman reaction associated with bi-lateral ear haemorrhage, thrombosis and deposition of "fibrinoids" thus lending support to the findings of Dannenberg and Scott (1958a) who thought that the glomerular lesions in hamsters dying of acute melioidosis resembled those described for the generalised Schwartzman phenomenon (Thomas and Good, 1952).

(g) Diagnosis.

The diagnosis of melioidosis has depended upon the recovery of the organism in culture or by animal inoculation, initial identification by conventional morphological, cultural and biochemical tests and finally, by pathogenicity tests in susceptible laboratory animals (rodents). Occasionally, but more often in recent years, attempts have been made to diagnose the disease by means

of allergic tests - the melioidin test (Olds and Lewis, 1954; Laws, 1967) or mallein test (MacLennan, 1953) - or by serological means. A number of serological tests have been used for this purpose in human disease, and in a few instances, in domestic animals. These are the agglutination test (Stanton and Fletcher, 1925b; Verge and Pairemaure, 1928; Blanc, Delage and Martin, 1943; Legroux and Blanc, 1943; Grant and Barwell, 1943; Harries et al., 1948; Cravitz and Miller, 1950; Brygoo, 1953a; Olds and Lewis, 1954; Nguyen-Ba-Luong, 1956; Fournier and Chambon, 1958 and Khaira et al., 1959), the indirect (passive) haemagglutination test using the protein antigen or the polysaccharide complex (Boyden, 1950; Fournier, Lajudie and Chambon, 1953; Nigg, 1963; Ileri, 1965; Laws, 1967; Spotnitz et al., 1967; Levine and Whelan, 1968; Strauss et al., 1969; Malizia et al., 1969; Alexander et al., 1970; Kaufmann et al., 1970; Kishimoto et al., 1971; Chofnas, 1972 and Clayton et al., 1973), the fluorescent-antibody inhibition test (Moody, Goldman and Thomason, 1956; Thomason, Moody and Goldman, 1956; Kishimoto et al., 1971) and the complement-fixation test (Stanton and Fletcher, 1925b; Cravitz and Miller, 1950a; Fournier et al., 1953a; Fournier and Chambon, 1958; Darby and Mendez, 1960; Nigg and Johnston, 1961; Cook, 1962; Nigg, 1963; Laws, 1967; Spotnitz et al., 1967; Levine and Whelan, 1968; Alexander et al., 1970). Although some of these tests have given useful results, all have shown a lack of sensitivity or specificity or both. It has been observed

that agglutinins are slow to develop in melioidosis (Stanton and Fletcher, 1925b; Brygoo, 1953; Laws, 1967b) and are found to a high titre in normal persons in non-endemic areas (Cravitz and Miller, 1950b) as well as in endemic areas (Brygoo, 1953a). Harries et al. (1948) testing the sera of patients suffering from melioidosis and, as controls, sera drawn from 56 patients affected with other infections (syphilis, tuberculosis, infective hepatitis and other fevers), found that none of the control sera gave a titre exceeding 1:20. They considered an agglutinating titre of 1:40 to be significant and a titre of 1:80 to be diagnostic of the disease. Fournier et al. (1953a) considered a titre of 1:80 to be significant since low antibody titres in melioidosis were observed in the sera of patients other than those with melioidosis, particularly in persons with Salmonella infections. Anti-whitmori serum (prepared in sheep) has also been shown to cross-agglutinate with Ps.pyocyanea (Legroux and Blanc, 1943 and Blanc, Delage and Martin, 1943) and Pf.mallei (Stanton and Fletcher, 1925b; Verge and Pairemaure, 1928; Legroux and Blanc, 1943; Cravitz and Miller, 1950a,b; and Fournier, 1967). It has also been found that agglutination tests give false-negative reactions in proven cases of melioidosis (Stanton and Fletcher, 1925b; Fournier et al., 1953a; and Brygoo, 1953a). Nguyen-Ba-Luong (1956) examined the sera of 50 apparently healthy pigs in a herd where an epizootic of melioidosis had occurred. Agglutinin

titres of up to 1:80 were found but the results indicated little or no correlation with the complement-fixation and haemagglutination titres except for a few in which an agglutinating titre of 1:80 corresponded to a moderately high haemagglutinating titre. He considered an agglutinating titre of 1:80 to be significant in melioidosis.

The complement-fixation test has also lacked specificity or sensitivity. Verge and Pairemaure (1928) showed that the serum of a glandered horse fixed complement to the same extent in the presence of either B.mallei or B.whitmori antigen. Cravitz and Miller (1950b) found that the serum from a case of human glanders would cross-react with M.pseudomallei though the titre was less than in the CF test with an M.mallei antigen. Cravitz and Miller (1950a) also observed cross-reactions in CF tests when rabbit anti-pseudomallei sera were tested against some but not all of their strains of M.mallei and vice versa. Fournier et al. (1953a) using as antigen an extract (Boivin's method) studied 31 culturally positive cases of melioidosis and found the CF test to be the least specific of three tests (agglutination, haemagglutination and complement-fixation). Prevatt and Hunt (1957) found M.mallei antigen cross-reacting with serum from a chronic case of human melioidosis. Cook (1962) in a serological survey for melioidosis in man and animals used a cell-free antigen and found only one of 619 persons reacting to the complement-fixation test with a titre of 1:10. A very small proportion of animals were also found

to have antibody titres but these were also in the range of 1:10 to 1:20 and rarely to a titre of 1:80. It has also been stated that some of the marsupial sera examined gave false-positive results when unclarified (non-centrifuged) antigen was used but centrifuged supernate had given negative results in all except three cases. Similarly, some of the marsupial sera had cross-reacted with unclarified Ps.aeruginosa antigen but all such sera had given negative results when tested with a clarified antigen of Ps.aeruginosa. Cook (1962) has stressed the need for the use of centrifuged supernate (cell-free antigen) in the complement-fixation test. Laws (1967) and Nigg and Johnston (1961) found the complement-fixation test using a cell-free antigen to be more specific and sensitive in detecting antibodies in human melioidosis and in the sera of experimentally infected monkeys, sheep and rabbits. Laws (1967) also using a cell-free antigen found a good correlation between complement-fixation test results and bacteriologically proven cases of melioidosis in man, goats and sheep. However, false-negative reactions were observed in bacteriologically proven cases of melioidosis in domestic animals including naturally infected goats, sheep and pigs (Laws, 1967) and false-positive reactions in humans (Fournier et al., 1953; Fournier and Chambon, 1958 and Alexander et al., 1970) found Ps.pseudomallei antigens cross-reacting with diverse anti-bacterial sera from hyperimmunised rabbits and human patients although they found excellent

specificity when evaluated with normal sera from humans. They found the complement-fixation test giving reactions in the sera of 5 of 11 patients with Ps.aeruginosa or Ps.stutzeri infections compared with reactions in the sera of 7 of 96 patients with other diseases. They suggested that the complement-fixing antigen contained a component common to some but not all strains of Ps.aeruginosa. The indirect haemagglutination (IHA) test using a protein antigen (tanned red blood corpuscles sensitised with antigen) was found unsatisfactory because of a lack of specificity. The IHA test using a purified polysaccharide antigen (prepared according to the method of Westphal, 1952) was found to be more sensitive than the complement-fixation test in detecting antibody referable to melioidosis in an apparently healthy population group (Nigg, 1963) in an endemic area and in experimentally infected rabbits. Laws (1967a,b) using a centrifuged supernate of a heated Ps.pseudomallei suspension as antigen found the IHA test to be as sensitive as the complement-fixation test in demonstrating significant antibody titres in natural cases of melioidosis in sheep, goats and humans and to be superior to the complement-fixation test in detecting antibody in affected pigs and cattle. (The complement-fixation test using guinea-pig complement has been found to give no reaction with anti-mallei sera (Hoet, Blomfield and Coombs, 1954)). The IHA test also revealed more reactors than the complement-fixation test among goats and sheep in farms where the infection had been recorded. However, Laws (1967a,b)

also found false-negative reactions in bacteriologically proven cases of melioidosis in sheep, pigs and a goat. Alexander et al. (1970) found the IHA test more specific than the complement-fixation test in that reactions were rare in normal human sera and in sera from various other human patients and hyperimmunised rabbits. However, variable negative reactions in proven cases of melioidosis were more often encountered with the IHA test than with the CF test and such false negatives were commonest in cases with localised forms of the disease. Fournier et al. (1953a) found the IHA test (antigen extracted by the Boivin procedure) the most promising of their three serological procedures but they too noted false negative reactions in human cases with the localised form of the infection. Boyden (1950) showed that mallein could haemagglutinate equine red blood corpuscles (RBC) in the presence of serum from a patient suffering from melioidosis. Nguyen-Ba-Luong (1956) found poor correlation between complement-fixation, haemagglutination and agglutination titres in serum samples of in contact pigs in a farm where melioidosis had been diagnosed. Similar observations were also recorded by Laws (1967b) in pigs from which Ps.pseudomallei was isolated. A poor correlation between the IHA and the CF test was observed in proven cases of human melioidosis (Alexander et al., 1970) and in the sera of persons in endemic areas (Nigg, 1963). It has been suggested therefore that both tests be applied in the diagnosis of melioidosis (Alexander et al., 1970) and in serological surveys (Nigg,

(1963).

The melioidin test was considered to be of value in the diagnosis of the disease in goats (Olds and Lewis, 1954; Laws, 1967b) but false negative reactions were observed in two culturally positive cases of melioidosis in goats (Laws, 1967b). These investigators also found either a rise in agglutinin titre (Olds and Lewis, 1954) or in complement-fixing antibody titres (Laws, 1967b) following the inoculation of melioidin in the melioidin test.

Some of the discrepancies observed in the serological diagnosis of melioidosis may be due to the different methods of preparation used, some of which have lacked a standardisation of reagents. The lack of specificity may be due also to antigenic components shared with other bacteria and it is possible that a lack of sensitivity of tests as noted in some findings may be due to the use of heterologous strains of Ps.pseudomallei in the preparation of antigens. Serological heterogeneity among strains of Ps.pseudomallei has been noted by a few workers (Alexander, Griffin and Gochenour, 1955; Fournier and Chambon, 1958; Chambon, 1960; Laws, 1967a,b and Dodin and Fournier, 1970).

(h) Treatment.

Even with the range of antimicrobial therapeutic agents now available the treatment of melioidosis, particularly the acute form, has presented problems (Alain et al., 1949; Levine and Whelan, 1968; Jackson et al., 1972) and in some cases, has ended in failure

(Montgomery, 1953; Baumann et al., 1967, Brundage et al., 1968). The delay in diagnosing the disease (in its many forms) or the identification of the causative agent (through lack of familiarity) might account for a number of the failures. Most of the cases reported in earlier times were diagnosed when the patients were either moribund or developing extensive lesions. In animals, it has invariably been diagnosed post-mortem.

Over a number of years, the published reports on human melioidosis have indicated the marked resistance of Whitmore's bacillus to a number of commonly used antibacterial agents but, apart from in vitro antimicrobial sensitivity tests (Mirick et al., 1946; Harries et al., 1948; Gutner and Fisher, 1948; Cruickshank, 1949; Green and Mankikar, 1949; Cros and Demarchi, 1950; Dunlop, 1952; Ives and Thomson, 1953; Brygoo, 1953b,c,d; Chambon et al., 1954; Prevatt and Hunt, 1957; Fournier and Chambon, 1958; Moustardier, Dulong De Rosnay and Salvat, 1959; Borschardt et al., 1966; Patterson et al., 1967; Brundage et al., 1968; Green and Tuffnell, 1968; Hobby and Lenert, 1968; Farkas-Himsley, 1968; Franklin, 1969; Hobby, Lenert, Maier-Engallena and DeNoia-Cicenia, 1969; Grunberg, Beskid, Delorenzo and Titsworth, 1969; Salisbury and Likos, 1970; Beaumont, 1970; Fisher, Hillegas and Naziri, 1971; Konopka, Lewis and Stieglitz, 1970; Eichoff, Bennett, Hayes and Feeley, 1970; Alexander and Williams, 1971; Bassett, 1971; Franklin, 1971

Chofnas, 1972 and Calabi, 1973) and a few *in vivo* studies on experimentally infected laboratory hamsters (Miller, Pannell and Ingalls, 1948) and mice (Hezebicks and Nigg, 1958; Khundanov, Devyatova, Padalko, Luk'yanova and Shkurko, 1961; Grunberg *et al.*, 1969 and Hobby *et al.*, 1969), the findings have been based on the experience of clinicians.

Chloramphenicol and tetracycline have remained the antibiotics of choice for use in human beings A few reports have also indicated the usefulness of sulphonamide drugs (Grant and Barwell, 1943; Harries *et al.*, 1948 and Green and Mankikar, 1949). The emergence of strains resistant to some of these antibacterial agents has been mentioned (Fournier and Chambon, 1958; Duong-Hong-Mo and Tran Hiap-Cuong, 1967). Relapses after an apparent response or cure have been recorded (McDowell and Varney, 1947; Alain *et al.*, 1949; Prevatt and Hunt, 1957; Diamond and Pastore, 1967). In recent years, kanamycin and novobiocin have also been used in the treatment of human cases. Some physicians have advocated therapy with massive, unconventional doses of these two antibiotics and chloramphenicol, either alone or in combination, over long periods (Cooper, 1967; Diamond and Pastore, 1967; Weber *et al.*, 1969 and Thin *et al.*, 1970) but others have considered such doses to be unwise and unwarranted (Spotnitz *et al.*, 1967; Flemma *et al.*, 1969 and Byrd and Puritz, 1970).

Possibly because melioidosis in domestic animals

often remains undiagnosed during life, treatment schedules have not been defined and rarely has there been an attempt to study the antibiotic sensitivities of strains of Ps.pseudomallei isolated from domestic animals. Retnasabapathy (1959), using Evans' Sentest tablets reported on the sensitivity of 2 strains of Whitmore's bacillus isolated from 2 pigs. Laws (1964), also using such tablets and Oxoid Multodisks, reported on the sensitivities of organisms isolated from a sheep and a bovine animal.

Part 2: The characters of Pseudomonas pseudomallei and its identification in the clinical laboratory

1. INTRODUCTION

Whitmore (1913) describing melioidosis in man indicated that the causative agent differed in a few morphological and cultural characters from the organism of glanders but bore many resemblances to it in its pathological features. He therefore proposed the name Bacillus pseudomallei. Thus the identity of the organism, based as it was on some common morphological and cultural characters was linked primarily to its recovery from glanders-like lesions thereby making the disease one of its classifying features.

Stanton and Fletcher (1921) describing new forms of the disease in man and laboratory animals, considered the name Bacillus pseudomallei to be invalid and proposed the specific name Bacterium whitmori to honour Colonel Whitmore.

For several decades however the taxonomic position of the organism of melioidosis remained obscure as that of the organism of glanders. Bacillus mallei has been variously classified. Owing to a lack of a suitable generic home, B.pseudomallei was often placed in the various genera into which B.mallei has been assigned from time to time. Thus, in the past, the organism of melioidosis has been referred to as Pfeifferella whitmori (Verge, 1928; Topley and Wilson, 1929; Finlayson, 1944; Wilson and Miles, 1955), Loefflerella whitmori (Brindle and Cowan, 1951; Minett, 1959; Wilson and Miles, 1964), Malleomyces pseudomallei (Bergey et al., 1939; Breed,

et al., 1948). Occasionally, it has been referred to as Whitmorella pseudomallei (Brygoo, 1957), Actinobacillus whitmori (Haupt, 1957; Soltys, 1963) and even as Mycobacterium pseudomallei (Rafyi and Mir Chamsy, 1952), and Flavobacterium pseudomallei (Weber et al., 1969).

The biological and some cultural affinities of Whitmore's bacillus to Pseudomonas aeruginosa were pointed out by a number of workers (Pons, 1927; Legroux and Kemal Djemil, 1931; Legroux and Genevray, 1933 and Legroux and Blanc, 1943). Brindle and Cowan (1951) in their studies on the morphology of 8 strains of Whitmore's bacillus, demonstrated polar flagellation and thought that the type of flagellation warranted the inclusion of this organism in the family Pseudomonadaceae. Lajudie, Fournier, Chambon (1953) confirmed these observations and agreed that Whitmore's bacillus, along with the organism of glanders, should be placed in the Pseudomonadaceae but they favoured the generic name Malleomyces to the proposed name Loefflerella. Wetmore and Gochenour (1956), in a more extensive study of Malleomyces and Pseudomonas, using 15 strains of M.pseudomallei, 3 strains of M.mallei, 20 strains of Ps.aeruginosa and 8 other strains of Pseudomonas demonstrated on electron micrographs the similarity in the flagellation of M.pseudomallei and Ps.aeruginosa and the absence of flagella in M.mallei. In addition, they showed that a number of cultural and biochemical characteristics were shared by Malleomyces and Pseudomonas. Therefore these workers too supported the inclusion of

M.pseudomallei in the Pseudomonadaceae. Brygoo (1957) whilst pointing out the similarity of some B.whitmori strains to Xanthomonas, indicated agreement to the proposal to classify B.whitmori in the Pseudomonadaceae but showed a preference to place it as a separate genus Whitmorella with Whitmorella pseudomallei as the type species. He also suggested the exclusion of the glanders organism from the Pseudomonadaceae; that it may remain as M.mallei in the family Parvobacteriaceae.

The close similarity of the organism of melioidosis and glanders to the aerobic pseudomonads as shown by many of these workers has led to the present designation of Whitmore's bacillus as Pseudomonas pseudomallei and glanders bacillus as Pseudomonas mallei (Breed, Murray and Smith, 1957). The more recent findings of Stanier, Palleroni and Duodoroff (1966) in their taxonomic studies on the genus Pseudomonas and those of Redfearn, Palleroni and Stanier (1966) in their comparative study of 26 strains of Ps.pseudomallei and 15 strains of Bacillus mallei have fully supported the current classification of Whitmore's bacillus and B.mallei under the 'pseudomallei' group of aerobic pseudomonads. The closer relationship that exists between the organism of glanders and the organism of melioidosis has also been shown through serological studies (Cravitz and Miller, 1950) and by bacteriophage affinities (Smith and Cherry, 1957). The findings of Mandel (1966) on the DNA base composition of the two species within the 'pseudomallei' group and, the demonstration of the hybridisation of the DNA of

Ps.pseudomallei and Ps.mallei (Rogul, Brendle, Haapla and Alexander, 1970) have left little doubt as to the genetic homology of the two species.

Although the taxonomic position of the organism of melioidosis has been made clear and characteristics to speciate Ps.pseudomallei have been defined (Redfearn et al., 1966), the methods adopted in diagnostic laboratories for the detection, isolation and the identification of the organism of melioidosis have changed little from those used many years ago (See: Appendix C). This may be due in part to the paucity of literature available to workers in the tropics and in part to the difficulty of introducing methods that are not conventional in clinical laboratories. Besides, in clinical laboratories, there is a need to apply technically simple tests that yield the most useful information in the shortest time. Presumably due to these drawbacks, a great deal of reliance has been placed on the characterisation tests for Whitmore's bacillus as laid down in standard veterinary and medical textbooks.

However, textbook versions appear to be not without anomalies that could lead to some confusion in the identification of Ps.pseudomallei. Current texts (Stableforth and Galloway, 1959; Soltys, 1963 and Wilson and Miles, 1964) describe the organism of melioidosis under one of the other of its many older generic names and the distinctive characters of the organism have been drawn to a greater part from the literature pertaining

to the similarities and dissimilarities between Whitmore's bacillus and the bacillus of glanders. This has tended to undermine the close similarities between Whitmore's bacillus and Ps.aeruginosa which unfortunately has also been characterised to emphasise its similarities and dissimilarities with Pseudomonas fluorescens.

The close similarities between Ps.pseudomallei and Ps.aeruginosa can be judged by perusal of the literature on Ps.aeruginosa. This organism is known to be a soil organism occasionally pathogenic to man. It is not known to give rise to contact infections and is regarded typically as a hospital pathogen which establishes infection in subjects with a severely lowered resistance. The usual portals of entry are cuts, burns or through surgery. The clinical importance that it has assumed in recent years has been because of its resistance to antibiotic therapy. Even although advanced nations have come to regard Ps.aeruginosa as an important pathogen only in recent years, this may not have been the case in the tropics. Pons (1927) stated that Ps.aeruginosa is especially pathogenic in the tropics where it may be responsible for typhoid-like infections and abscesses in the liver. Lusis and Soltys (1971) in a review of literature on Ps.aeruginosa have stated that the diseases caused by this organism in animals are somewhat similar to those in man - suppurative processes and occasional generalised infections - and have listed the many different animal species, including

domestic animals, in which infections have been recorded. It is also known that laboratory rodents are susceptible to both natural and experimental infections of Ps.pseudomallei and that lesions including the Strauss-type reaction seen with the organisms of melioidosis (Whitmore, 1913b; Knapp, 1915; Stevenson, 1916; Stanton and Fletcher, 1925a; Cox and Arbogast, 1945; Robinson and Ballion, 1966; Montgomery, 1963; Wilson and Miles; 1964) and glanders have been observed.

Although chromogenicity - the ability to produce phenazine compounds - has often been emphasised as an important character of Ps.aeruginosa, a number of investigators (Hadley, 1927; Gaby, 1946; Haynes, 1951; Gaby and Free, 1953; Sutter, 1968 and Gilardi, 1968) have reported on the occurrence of achromogenic strains in primary cultures and also in secondary cultures through loss of chromogenicity in the originally pigmented strains.

Gaby and Free (1953) stated that the identification of Ps.aeruginosa regardless of its pigmenting property can be made on the basis of its (1) variable but typical colony, (2) odour of trimethylamine, (3) Gram-negative reaction, motility and the production of a pellicle in liquid media, (4) acid production in glucose but not in lactose and sucrose, (5) liquefaction of gelatin, (6) inability to hydrolyse urea. Haynes (1951) stated that apyocyanogenic strains of Ps.aeruginosa could be identified by methods that depend on correlated characteristics such as (a) the ability to grow at $41^{o}\pm 1^{o}C$, (b) the ability

to oxidise potassium gluconate, in shaken culture, to a reducing compound presumed to be 2-ketogluconate and (c) the production of slime in static culture in a medium containing potassium gluconate as the principal carbon source. However, Wetmore and Gochenour (1956) showed that apart from the odour of trimethylamine and the ability to oxidise gluconate, all the other characteristics listed by Haynes (1951) and Gaby and Free (1953) for the identification of achromogenic strains of Ps.aeruginosa were properties shared by Ps.pseudomallei. Furthermore, they found a number of other commonly used biochemical tests (nitrate reduction, production of hydrogen sulphide, production of indole, utilisation of citrate, hydrolysis of urea, proteolysis and the oxidation of carbohydrates) to be of little or no value in the differentiation of the two species and therefore recommended the use of growth inhibition tests (inability to grow on Salmonella-Shigella agar, Desoxycholate agar, sodium azide agar, cetyltrimethyl ammonium bromide agar and the inability to grow at a temperature of 21^{o}C) to differentiate Ps.pseudomallei from Ps.aeruginosa.

Despite these findings, the methods used in the identification of Ps.pseudomallei in clinical laboratories in general, have changed little. Apart from the publications of Sutter (1968) and Gilardi (1968 and 1969) who included some of these tests in their studies on pseudomonads including Ps.pseudomallei, the publications of other research workers do not show such attempts. The practice has been to identify the organism on the

basis of its popularly known and perhaps commonly acknowledged morphological and cultural characters (bi-polar staining, wrinkled colonies, a characteristic odour and a metallic sheen when grown on solid media) and on biochemical characters that were primarily designed for the identification of 'coliforms'. This conventional approach to identification is evident even in several recent publications (Joy et al., 1960; Montgomery, 1963; Rimington, 1962; Egerton, 1963; Robinson and Ballion, 1966; Laws, 1964; Magee et al., 1967; Lim and Retnasabapathy, 1967; Ketterer and Bamford, 1967; Baumann and Morita, 1967; Chooi et al., 1967; Spotnitz et al., 1967; Green and Tuffnell, 1968; Stokes and McCarthy, 1969; Brundage et al., 1968; Weber et al., 1969; Greenawald et al., 1969; Salisbury and Likos, 1970 and Osteraas et al., 1971), some of which have also indicated a reliance on the pathogenicity to laboratory animals ('Strauss reaction' in guinea-pigs) as one of the principal criteria in identifying the species. It would therefore seem that the identification of Ps.pseudomallei has largely depended on a number of loosely fitting characters. Strains of low virulence to laboratory animals may escape detection.

Apart from these considerations, the colonial heterogeneity reported in both primary and secondary cultures may present difficulties in identifying the organism. Instances may occur where genuine strains of Ps.pseudomallei could be mistakenly disregarded as 'contaminants' because of atypical colony appearances.

Colony dissociation or variation in Ps.pseudomallei has been described by some workers (Stanton and Fletcher, 1925a and 1927a; Nicholls, 1930; Souchard, 1932; Finlayson, 1944; Cottew, 1950; LeGac et al., 1954; Beamer et al., 1954; Nigg, Ruch, Scott and Noble, 1956; Chambon and Fournier, 1958 and Zierdt and Marsh, 1971), though some of these descriptions appear to be at variance with one another. Nevertheless, variations in colonial morphology can lead to confusions not only in the detection and identification of the organism but also in the repeatability of experiments for, as in other bacteria, these differences in colonial morphology may be associated with differences in virulence, antigenicity, susceptibility to chemotherapy and in other properties such as stability on storage, biochemical features etc. Differences that can be correlated to colony types have been mentioned by some workers. Stanton and Fletcher (1925a and 1927) found their 'corrugated' and 'ultracorrugated' colonytypes to be more virulent than their 'mucoid' type. A number of investigators (Nicholls, 1930; Finlayson, 1944; Beamer et al., 1954) found their 'rough' strains to be more virulent than their other types. Nigg et al. (1956) obtained an extremely virulent, small, rough, yellow colony from a relatively less virulent "rough, lacy" type colony by serial passage in mice. Biochemical activity (including the breakdown of carbohydrates) was found to be more marked in 'rough' colony types than in 'smooth' colony types by some investigators (Nicholls,

1930; Finlayson, 1944). Beamer *et al.* (1954) found their 'smooth' (S) type colonies to be more haemolytic than their 'rough' (R) type colonies. Nicholls (1930) also described a mucoidal type of colony which he considered as a "suicidal" type because of a tendency to die out in culture within 10 days.

The cultures used in the present work are those already identified as the organism of melioidosis by the donor institutions. Many of the cultures are old laboratory stock strains. Some of the donor laboratories are those based in endemic areas where facilities for extensive laboratory investigations may be lacking. A few of the many strains received from non-endemic areas (U.S.A. and the U.K.) were from isolations made in clinical laboratories in the West where facilities may have been extensive but which may have lacked in personnel who have had experience in the identification of the organism. Misidentifications cannot be ruled out therefore for there is at least one publication on melioidosis (Garry and Koch, 1951) where the colonies of the organism have been described as greenish coloured, producing a diffusible pigment. The organism identified by Garry and Koch (1951) was subsequently shown by Haynes (1951) to be a strain of *Ps.aeruginosa*. There is also an instance where the organism was mistakenly interpreted as *Ps.aeruginosa* (Poe, Vassallo & Dorn, 1971). *Ps.pseudomallei* can also be misidentified as *Alkaligenes faecalis* (Borchardt *et al.*, 1966; Gutner and Fisher, 1948).

As a preliminary step to our studies, it was

therefore considered desirable to re-investigate some of the morphological, cultural and biochemical characters by which Whitmore's bacillus has been recognised in the clinical laboratory, and, at the same time apply some of the tests suggested by Wetmore and Gochenour (1956) to evaluate their usefulness. The range of nutritional tests suggested by Redfearn *et al.* (1966), however desirable they may be, were not included because such tests cannot be easily accommodated within clinical laboratory diagnostic schema. On the other hand, the suggestions (Brundage *et al.*, 1968; Gilardi, 1971 and Zierdt and Marsh, 1971) that strains of *Ps.pseudomallei* are a uniform group in their sensitivity to antimicrobial agents and that their sensitivity pattern may serve as an aid in the identification of the organism, were considered worthy of investigation. Animal pathogenicity tests using live organisms were not attempted because of attendant dangers but an attempt using sterile culture filtrates was made to detect the production *in vitro* of toxins first reported by Colling, Nigg and Heckly (1958).

2. MATERIALS AND METHODS

(a) Cultures employed

Cultures of Ps.pseudomallei isolated from man, several domestic species and a few wild animals held in captivity, were obtained from medical and veterinary laboratories in South East Asia, Australia, Papua and New Guinea, the U.S.A., France and the U.K. Data including the source and date of isolation (where available) of the 118 isolates of Ps.pseudomallei obtained from these laboratories, are given in Appendix A. Two of these cultures on receipt were found to be non-viable. All but one of the strains received from laboratories in the U.K. were National Collection Type Culture (NCTC) strains. Eight of the isolates from Sabah, Malaysia, were those isolated by the author towards the latter part of the year 1969, and had not been subcultured except for the procedures involved in the initial purification before lyophilisation and dispatch to the U.K. All other cultures from Sabah were isolated in 1970 and these too had not been sub-cultured except for the purification procedures before dispatch to the U.K. Cultures from Sabah, can therefore be regarded as the most recently isolated strains in the collection and as strains which had been subjected to the minimum of sub-culture passages.

In referring to these isolates, the term 'strain' has often been used but this is not to connote genetic differences. It has been used as a substitute for the

term isolate.

Six strains of Ps.aeruginosa including a NCTC strain and a pyocin producer (strain P10) were used in comparative studies on some of the cultural and biochemical properties of Ps.pseudomallei strains. Four of these strains were also used in antimicrobial disc-sensitivity studies to compare their sensitivity patterns with those of Ps.pseudomallei strains. Histories of these strains are also included in Appendix A.

(b) Culture media and the temperature of incubation

The media used have been stated under each experiment. Unless otherwise stated, all incubations were carried out at 37°C.

(c) Preparation and preservation of lyophilised stock cultures

Ninety-eight of the 118 strains of Ps.pseudomallei were received at this laboratory as lyophilised cultures, the rest were received as slope cultures. Within a day or two of receipt of a culture, it was plated on to a nutrient agar plate, incubated for 36 hours and the inoculum from a single colony representative of the predominant colony type was re-plated on to a nutrient agar plate. This plate was also incubated for 36 hours and the inoculum, once again from a single colony was spread on to a nutrient agar slope. The slope was incubated overnight. The scraping from the overnight growth was suspended in a small volume of nutrient broth

and the suspension was seeded on to a set of 2 nutrient agar plates (or 'medical flats'). The set of plates was incubated overnight and the growths of the plates were scraped off and suspended in 2-2½ ml of normal horse serum. Aliquots (0.1 ml approx.) of this suspension were dispensed into lyophilising tubes and these were dried and sealed under vacuum.

The slope culture mentioned above was used to carry out all the preliminary tests (Cowan and Steel, 1965, p76) to satisfy that the lyophilised culture was that of a Gram-negative, motile, aerobic, non-fermentative bacterium. In many cases, the same slope culture, kept at room temperature, was used within one month of culture to test for the differential characters of Ps.pseudomallei as listed by Cowan and Steel (1965, p.81). Strains that conformed to this description (barring starch hydrolysis and acid production in mannitol) were considered as Ps.pseudomallei and the lyophilised stock cultures of these will be referred to as the "primary" strains to differentiate them from the secondary strains - the dissociants - encountered in the course of subsequent subculture.

(d) Stock slope cultures (Working cultures)

For the day-to-day experiments, subcultures were drawn from a stock nutrient-agar slope culture (working culture) which was prepared from the lyophilised primary strain or from a dissociant strain, depending on the requirement. These cultures were incubated overnight

and were then left at room temperature for use as and when required. The majority of such stock cultures were usually found viable for many months except for some "mucoid" dissociants which lost their viability within days to weeks. However, stock slope cultures, unless required for viability studies during storage, were discarded within one month of preparation and were replaced with fresh working cultures. In the case of primary strains, fresh stock slopes were prepared from the lyophilised cultures but in the case of the dissociant types, a fresh stock slope of the dissociant was prepared by subculturing from the previous stock slope into a nutrient agar plate and picking a colony representative of the dissociant into a nutrient agar slope or broth. The preparation of stock slope cultures of mucoid dissociants was essentially similar to that adopted for other dissociants but subculturing was done at weekly or fortnightly intervals in order to minimise the chances of losing this variant during prolonged storage.

(e) Examination for colony characters

(i) Colony morphology was studied on nutrient agar (Oxoid) plates by the commonly applied 'plating' technique (Cruickshank, 1965, p.789) to obtain discrete colonies. The method was also extended to observe colony morphology on blood agar (5% Oxoid horse blood) and on MacConkey agar (Oxoid). At first the colony morphology was studied on plates incubated for 24 hours

but later the plates were incubated for 48 to 72 hours or longer to allow for the full development of colonies and to facilitate the detection of any dissociants.

(ii) Dissociative tendencies of 9 strains were studied by serial subculture of selected colony types from both mono-colony and multi-colony type "secondary" cultures. A colony was picked up and plated on to nutrient agar and was incubated for 72 hours. A single colony from this culture was replated to nutrient agar and was incubated for 72 hours. The procedure was repeated through 10 serial subcultures noting the number of occasions when dissociants were seen. Any dissociants that occurred during these transfers were subcultured in parallel for the remainder of the 10 serial passages and the number of times when dissociants were seen among them was also recorded.

(iii) Dissociants that may occur in broth cultures (with different pH levels) grown up to 72 hours were roughly estimated by picking a colony representative of a type, suspending it in a 2 ml volume of nutrient broth and inoculating a loopful (standard loop) of it into each of 3 x 10 ml volume broths adjusted to a pH of 7.0, 7.4 and 7.8. At 24, 48 and 72 hours incubations a loopful of each culture was withdrawn and suspended in 10 ml volume of broth. Each of these suspensions was further diluted (up to 10^{-5}) and 1 ml volumes of each dilution in the series was spread on to plates of nutrient agar which were then incubated for 72 hours. After this the plates showing dense populations but countable numbers

of colonies (150 to 200) were examined for dissociants. The percentage of dissociants was estimated.

(f) Pigment production in culture media

No special media were used in this study. Cultures on nutrient agar plates and slopes and in nutrient broth were grown overnight at 37°C and were then placed in a cupboard at room temperature (25°C approx.). Cultures on nutrient agar plates were examined daily for one week. Slopes and broth cultures were examined at intervals for up to a month.

(g) Viability of cultures in storage at room temperature 25°C and at 5°C

Colonies representative of predominant colony types of the 'primary' cultures and some of the other dissociants were grown overnight in nutrient agar slopes and were then held at room temperature for a period up to 3 years. At regular intervals - daily for the first week, weekly for the next 3 weeks, monthly for the first 6 months, quarterly to half-yearly intervals thereafter - these cultures were checked for viability by inoculating into nutrient broth or on to nutrient agar plates or slopes and incubating for 4 to 5 days at 37°C. Absence of turbidity in broth or visible growth on the solid media was interpreted as a complete loss of viability.

A batch of strains selected at random was also tested for viability during storage at 5°C. The cultures were tested as above up to a period of 3 months as cultures were found non-viable after that period.

(h) Pellicle growth in liquid media

Selected colony types from primary cultures were grown in nutrient broth (Oxoid, pH 7.4) in a static water bath at 37°C for 1 week and daily observations were carried out to note the development of pellicles.

(i) pH changes in nutrient broth cultures

pH changes in cultures were measured with a pH meter[1]. The recordings were made daily for 1 week. The cultures were those prepared from selected colony types from a number of primary cultures. A colony representative of the type was picked and suspended in about 2 ml of nutrient broth. A loopful each of this suspension was inoculated into 7 x 10 ml volumes of nutrient broth. These were incubated at 37°C and a 10 ml sample was withdrawn each day to measure the pH. It was discarded after the measurement.

(j) Haemolysis on blood agar

Cultures were grown on sheep and horse blood agar plates containing 5% erythrocytes which had been washed twice in sterile physiological saline before mixing with the blood agar base ('Oxoid'). Fresh batches of commercially available sheep blood ('Oxoid') and horse blood ('Oxoid') were used for obtaining the erythrocytes. A few strains were also studied on sheep blood agar incorporating freshly drawn blood from a healthy sheep at the laboratory. A few strains were also examined

[1]"Pye" Model 292 pH Meter, Pye-Unicam, Cambridge, U.K.

by placing a loopful of each culture at approximately equal distances on horse blood agar and sheep blood agar plates. Haemolysis of blood agar by a few strains of Ps.aeruginosa was also studied by this method.

(k) Motility and staining reactions

Specimens for the above studies were drawn from 16 to 18 hour nutrient broth cultures. Motility was checked by the 'hanging-drop' method using the ordinary light-microscope. Jensen's modification of Gram stain (Cruickshank, 1965, p.649) was used throughout the study. Bi-polar staining property was studied using Leishman's stain for some strains and Loeffler's methylene blue stain for others. Ziehl-Neelsen's staining procedure (Cruickshank, 1965, p.652) but omitting the alcohol-decolourisation step was applied to test for acid-fastness.

(l) Examination for capsulation and other surface appendages

These examinations were carried out in an electron microscope[1]. A 2% aqueous solution of phosphotungstic acid (PTA) pH 6.9 to 7.0 was employed for negative staining. Bacterial strains forsuch examinations were grown in 10 ml volumes of nutrient broth and, in later experiments, on nutrient agar slopes. Young cultures (12 to 18 hour growths) were used for the study of flagellation. The examination for capsulation was carried out on the same cultures as well as on those grown for 24 hours or longer. Some of the strains were also examined for capsulation by the wet India-ink method

[1]Model AEI-EM 6B, Associated Electrical Industries Ltd., Essex, U.K.

of negative-staining (Cruickshank, 1965, p.658).
For electron microscopy, broth cultures were centrifuged at low speed (2000-3000 r.p.m.) for 20 minutes in an MSE centrifuge[1] and the cell sediment resuspended in phosphate buffered saline (PBS) pH 7.2, to obtain a suitable cell density (10^{10} cells/ml approx.) for the examination. The organisms scraped off the nutrient agar slopes were suspended in PBS to a similar density but without the preliminary centrifugation. One drop of the cell suspension was mixed with an equal volume of the stain. Immediately afterwards, a collodion or carbon coated copper grid was placed with its coated face downwards on the surface of the stained suspension. After 30 to 60 seconds, the grid was lifted off with a pair of fine forceps or a bacteriological wire loop and the excess fluid was drained off by touching the periphery of the grid or the wire-loop holding the grid with a filter paper. The specimen was allowed to dry in the open for a further 3-5 minutes and was then examined in the electron microscope.

(m) Growth at different temperatures of incubation

The ability of strains to grow at 5°C, 21°C and 42°C was examined. A fan-blown, thermostatically controlled, refrigerated incubator[2] was used for growing cultures at 5°C and 21°C. Growth at 42°C was carried out in a shaking waterbath[3] (40 strokes/min.). Control cultures were incubated at 37°C. Nutrient broth in 10 ml volumes was maintained at the required

(1) MSE 18 Refrigerator Centrifuge
(2) "Hearson" model incubator, Astell Laboratory Service Co. London.
(3) Model SSB/4, Grant Instruments (Cambridge) Ltd., U.K.

temperature prior to inoculation. The inoculum consisted of a loopful of an overnight broth culture prepared from a representative colony of a primary culture or from a slope of a stock culture. All cultures were incubated for 7 days and were examined daily for visible growth and growth in comparison to the 'control' cultures.

(n) Biochemical and nutritional characteristics

Each strain of Ps.pseudomallei and a number of secondary dissociants was subjected to the following tests.

(i) production of catalase
(ii) production of oxidase
(iii) oxidation or fermentation (OF) of glucose
(iv) constitutive production of arginine dihydrolase
(v) starch hydrolysis
(vi) urea hydrolysis
(vii) proteolytic activity on gelatin and on Loeffler's serum
(viii) production of hydrogen sulphide
(ix) production of indole
(x) reduction of nitrate
(xi) oxidation of gluconate
(xii) utilisation of malonate
(xiii) utilisation of citrate
(xiv) oxidation of carbohydrates
(xv) methyl red test
(xvi) Voges-Proskauer test
(xvii) KCN (potassium cyanide) resistance
(xviii) action on litmus milk
(xvix) lecithinase activity
(xx) ability to grow on MacConkey agar (Oxoid), Desoxycholate citrate agar (Oxoid), Salmonella-Shigella agar (Oxoid) and Cetrimide agar
(xxi) growth on nutrient agar containing sodium chloride (2%, 3% and 4%)
(xxii) growth on bile salts agar (10% and 40% bile)
(xxiii) ability to grow under anaerobic conditions.

(i) Catalase production. The organism was streaked on to a nutrient agar plate and grown at 37°C for 24 to 36 hours. Two to three isolated colonies were then picked on to a clean microscope slide and, using a Pasteur pipette, a drop of hydrogen peroxide solution (10 volumes) was placed on this cell mass. The development of gas bubbles within one to five minutes was read as a positive reaction.

(ii) Oxidase activity. A few colonies picked off the nutrient agar plate culture used for the catalase test were smeared over the area of a filter paper moistened with a few drops of 1% tetramethyl-p-phenylene-diamine. The appearance of a dark purple colour within 10 seconds was read as a positive reaction.

(iii) Oxidation or fermentation (OF) of glucose. The medium prepared according to the method of Hugh and Leifson (1953) was dispensed in 5 ml volumes in tubes 1 cm x 10 cm plugged with cotton-wool, sterilised and stored in a refrigerator. Immediately before use, the medium was steamed to expel any dissolved air and cooled to solidify. Duplicate tubes of this medium were inoculated by stabbing with a straight wire. Melted soft paraffin (ca. 1 ml) was layered on to the surface of the medium in one of the two tubes. The tubes were incubated for a period up to 14 days and were examined daily for fermentative or oxidative reactions. Acid production in the open tube alone was interpreted as an

oxidative reaction.

(iv) Arginine decarboxylase. The method was according to the modification of the method of Møller (1955) described by Cruickshank (1965, p.824). The medium was inoculated with a straight wire through the paraffin layer. The tubes, including controls, were incubated and examined daily for 4 days. The development of a yellow colouration (acid production) followed by violet colouration was interpreted as due to the decarboxylation of arginine.

(v) Starch hydrolysis. The medium was prepared according to the method of Cowan and Steel (1965). Plates were inoculated and incubated at 30°C for 5 days. The plates were then flooded with Lugol's iodine diluted 1 : 5 in distilled water. A clear, colourless zone around or beneath the colonies was interpreted as due to the hydrolysis of starch.

(vi) Urea hydrolysis. The urea medium of Christensen (1946) was inoculated and incubated for 14 days. The slopes were examined daily for alkalinity.

(vii) Proteolytic activity. (a) Gelatin medium (Oxoid) was prepared according to the manufacturer's recommendations and was dispensed in 10 ml volumes in Universal bottles. The inoculum was stabbed into the medium with a straight wire. Some batches of cultures

were incubated for a period up to 7 days at 37°C and were examined daily after placing in a refrigerator for 10 to 15 minutes to set the gelatin. Other batches were incubated at room temperature for up to 14 days.

(b) Loeffler's serum was prepared in petri dishes according to the method of Cruickshank (1965, p.751) using horse serum. The plates were stab-inoculated with cultures so as to accommodate 5 strains to a plate at roughly equidistant points - 4 stabs radially and single stab centrally. The plates were incubated for a period of 10 days and examined daily. Well-formation at the site of inoculation was considered as liquefaction and clearing round the periphery of the inoculum was interpreted as due to a weak liquefaction.

(viii) Production of hydrogen sulphide. The organism was inoculated into nutrient broth and a filter-paper strip (5mm x 50mm) previously impregnated with a saturated aqueous lead acetate solution and dried, was placed between the mouth of the bottle and the cap so that one end of the paper strip hung freely in the bottle without coming into contact with the medium. The cap was screwed tight and the culture was incubated for a period up to one week. Blackening of the lead acetate strip indicated the release of hydrogen sulphide.

(ix) Production of indole. Peptone water (Oxoid) cultures in 10 ml volumes were grown at 37°C for 48 hours and then shaken with 1 ml of ether. After allowing the

ether layer to separate, 0.5 ml of Ehrlich's rosindole reagent (p-dimethylaminobenzaldehyde in acid-alcohol) was added. A rose-pink colouration in the ether layer indicated the presence of indole.

(x) Reduction of nitrate. The medium used in this test was prepared and tested according to the method given by Cowan and Steel (1965). A 10 ml volume nitrate broth was inoculated and incubated for 5 days. To 5 ml volume of this culture was added 1 ml of an acid solution of sulphanilic acid followed by an equal volume of an acid solution of α-naphthylamine. Development of a red colour was interpreted as due to the presence of nitrite. Those cultures that were found to be negative to this test were tested for the free nitrate by adding zinc dust at the rate of about 5 mg/ml to reduce the nitrate. The development of a red colour after the addition of zinc showed the presence of nitrate in the medium which had not been reduced by the organism but absence of a red colour indicated that all nitrate in the medium had been reduced by the organism beyond nitrite (i.e. to nitrogen).

(xi) Oxidation of gluconate. Gluconate broth was prepared according to the method of Shaw and Clarke (1955) and dispensed in 10 ml volumes. The medium was inoculated and incubated for up to 4 days. Aliquots of 1 ml were drawn at 48 and 72 hours. An equal volume of Benedict's reagent (sodium citrate 17.3 g.; Na_2CO_3,

anhydrous, 10 g.; $CuSO_4.5H_2O$ 1.73 g.; distilled water to 100 ml) was then added and the mixture was boiled in a waterbath for 10 minutes. The development of a green to orange precipitate was recorded as a positive result. Slime production was observed by swirling the gluconate broth culture. A ropy mass on swirling was interpreted as due to the production of slime.

(xii) Utilisation of malonate. A combined malonate-phenylalanine medium was prepared according to the method of Shaw and Clarke (1955) and dispensed in 3 ml volumes. It was inoculated and incubated for up to 72 hours. The development of a deep blue colour indicated the utilisation of sodium malonate. At the end of this period of incubation the culture was tested for the deamination of phenylalanine by acidifying it with 0.1 ml of 0.1N-HCl and adding 0.2 ml of a 10% aqueous solution of $FeCl_3$. The culture was then shaken and examined immediately for the appearance of a green colour.

(xiii) Utilisation of citrate. Koser's modified citrate medium (Cowan and Steel, 1965) was used in the study. The inoculum was made by touching the surface of a colony on nutrient agar with the point (tip) of a straight wire and transferring such inoculum by stirring the citrate medium with the tip of the wire.

(xiv) Oxidation of carbohydrates. Initially,

the organisms were grown in 5 ml volumes of peptone water containing bromothymol blue as indicator and 1% of the following substrates, dextrose, laevulose, mannose, galactose, arabinose, xylose, rhamnose, sucrose, maltose, lactose, trehalose, raffinose, inulin, dextrin, glycerol, mannitol, dulcitol, sorbitol, salicin, and inositol. The cultures, in loosely capped bottles, were incubated and examined daily for a period of two weeks.

In subsequent experiments, the medium of Hugh and Leifson (1953) and a modification of it substituting ammonium sulphate (2 g/litre) for peptone water in addition to a higher concentration of agar (2%), were used for testing acid production. The medium was poured into plastic disposable petri-plates (9 cm diameter) to a depth of approximately 0.6 cm. The cultures, 5 strains to a plate, were stab-inoculated at roughly equidistant points and were incubated. The plates were examined daily for a colour change around the stab growth for a period up to 14 days.

(xv) Methyl red (M.R.) test. The glucose-phosphate medium was prepared according to the method of Cowan and Steel (1965) and was dispensed in 10 ml volumes. The cultures were incubated for 4 days. The test was performed by adding 2 to 3 drops of a 0.25% alcoholic solution of methyl red to 5 ml of culture. The development of a yellow colour was interpreted as a negative reaction and a red colour as positive.

(xvi) Voges-Proskauer (V-P) test. A 2 ml volume of the 4-day glucose-phosphate culture prepared for the M.R. test was used. Barritt's (1936) modification was applied. 0.8 ml of a 5% alcoholic solution of α-naphthol and 0.3 ml of 40% caustic potash was added to the culture and shaken. The culture was examined after 5 minutes for any pink colouration indicating a positive result.

(xvii) KCN resistance. The medium used is that described by Cowan and Steel (1965) which is a modification of the original medium of Møller (1954). The KCN broth was dispensed in 5 ml volumes along with a batch of basal broth containing no KCN. These batches were held in storage at 5°C for use when required. Batches of media not used within 4 weeks of preparation were discarded. Cultures were incubated and were examined for growth (turbidity of medium).

(xviii) Action on litmus milk. The medium was prepared according to the method of Cowan and Steel (1965). The cultures were incubated for 14 days and were examined daily for colour changes (acidity or alkalinity) and clotting.

(xix) Lecithinase activity. The plate test as described by Cruickshank (1965, p.837) was used. Egg-yolk agar plates were stab-inoculated to accommodate 8 strains to a plate and were incubated up to 7 days. The plates

were examined daily for a lecithovitellin reaction. The plate was flooded with a saturated aqueous solution of copper sulphate, allowed to stand for 20 minutes, drained and dried and then examined for lipase activity. A greenish-blue appearance of the opalescent zone was interpreted as due to a lipase activity. The absence of such a colour characteristic on the opalescent zone was interpreted as a positive lecithovitellin reaction.

(xx) Growth on MacConkey agar, Desoxycholate citrate agar (DCA), Salmonella Shigella agar (SS agar) and Cetyltrimethyl ammonium bromide (CTAB) agar. MacConkey agar, DCA and SS agar were prepared according to the manufacturer's (Oxoid Ltd.) instructions. The method of Wetmore and Gochenour (1956) was used in preparing the CTAB agar medium. Petri plates prepared with such media were stab-inoculated to accommodate 8 strains to a plate. In a few experiments, 10-fold serial dilutions of washed bacterial cells grown overnight in nutrient broth were prepared and counts made by the technique of Miles and Misra (1938). Plates were incubated for 3 days and examined daily for growth in the stab-inoculated plates and for viable colonies in the Miles and Misra plates. Nutrient agar plates were used as 'controls'.

(xxi) Growth on nutrient agar containing sodium chloride. The effect of sodium chloride was determined using plates containing ox-heart infusion agar with

varying concentrations of NaCl - 2%, 3% and 4%. The infusion broth was prepared according to the method of Cruickshank (1965, p.740) to which agar was added to a concentration of 1.2%. The plates were stab-inoculated and incubated for 7 days and examined daily for growth.

(xxii) Bile tolerance. Bile salts agar containing 10% and 40% bile were prepared according to the method described by Cowan and Steel (1965). Plates were stab-inoculated and incubated for 7 days and examined for growth.

(xxiii) Anaerobic growth. Glucose broth and nitrate broth were prepared according to the method described by Cowan and Steel (1965). The broths were pre-heated to expel dissolved oxygen and cooled to 40°C immediately before inoculation. Oxoid nutrient agar was used in the plate cultures. The broth cultures in McCartney bottles and the plate cultures were placed in anaerobic jars and incubated for 3 days before examining for growth. The caps of bottles were kept loose during incubation.

(o) Toxins and 'soluble' haemolysins

Nine strains (4-SR; 15-M; 32-R; 33-SR; 42-S; 108-S; 111-SR; 74-S and 118-SR) known to produce varying degrees of haemolysis or no haemolysis on horse blood agar were used for the study of haemolysin activity

of broth culture supernates (crude supernates) and of extracellular material obtained by growth on cellophane-layered tryptone soya agar. Aliquots of some of these preparations boiled to 100°C for 15 minutes were also used for testing toxicity for mice and for observing any skin reactions in a rabbit inoculated intradermally.

(i) Preparation of crude culture supernates. A single colony of a strain picked from a 72-96 hour plate-culture was suspended in approximately 2.5 ml of tryptone soya broth (Oxoid, CM129) and 1 ml was inoculated into each of two 10 ml volumes of tryptone soya broth. One of the two broth samples was incubated at 37°C for 3 days while the other was incubated for 6 days. At the end of the incubation period the culture was centrifuged at 4000 r.p.m. for ½ hour. The supernatant fluid was collected and its pH adjusted to 7.3. It was then divided into two equal parts (approx. 4.5 - 5 ml), one of which was heated at 100°C for 15 minutes, the other being used unheated. Some trials were carried out using unheated preparations filtered through "Millipore" membranes (pore size = GS 0.22μ) or sterilised by adding chloroform (5% v/v).

(ii) Preparation of 'extra-cellular' material from cultures grown on cellophane-layered tryptone soya agar. Tryptone soya agar prepared from tryptone soya broth (Oxoid, CM129) by the addition of 1.5% agar was poured into large square petri-plates (10 cm x 10 cm) to a

standard depth (25 ml per plate). After the agar had solidified, a steam-sterilised film of cellophane ("Viskin") cut to the required size was overlaid so as to cover the whole surface of the agar. A colony picked from a plate culture was suspended in tryptone soya broth and made up to a 5 ml suspension in the same broth. An inoculum of 0.5 ml of the suspension was pipetted on to each of 10 cellophane-agar plates and spread with a sterile glass spreader. Five plates were incubated at 37°C for 3 days while the other five were incubated for 6 days. After incubation, the growth on each batch of five plates was pooled by washing the cellophane films in a 12 ml volume of normal saline contained in a large petri-plate using aseptic techniques. The saline washings were centrifuged at 4000 rp.m. for ½ hour. The supernate of each strain was divided into two equal parts, one of which was heated at 100°C for 15 minutes. The preparations were stored in the refrigerator if not used on the day of preparation. From each sample, 1 ml was drawn for testing the haemolytic activity. The remainder from the heated sample was used for animal inoculations.

(iii) Preparation of cell-extracts from cultures grown on tryptone soya agar. Several tryptone soya agar plates were seeded with a cell suspension prepared from a single colony of a selected strain and incubated for 72 hours. The growth was scraped off with the edge of a flamed and cooled microscope slide and the scrapings

were transferred to a small sterile plastic disposable petri dish (5 cm diam.) and weighed to obtain 3gm (wet weight) of cells. The cells were suspended in 5 ml of normal saline, transferred to a centrifuge tube and made up to 50 ml with saline. The cells were washed twice in similar volumes of saline by centrifugation at 4000 r.p.m. for ½ hour. The saline supernate at the end of the second washing was discarded and the cell sediment resuspended in 15 ml of distilled water. This suspension was frozen and thawed 10 times (over a 2-day period). The disrupted cell-suspension was centrifuged at 8000 r.p.m. for ½ hour to obtain the cell-free supernate which was divided into two equal parts and one part was boiled at 100°C for 15 minutes. A 1 ml volume was drawn from each part to test for haemolytic activity. The remaining volume of the heated cell extract was retained for animal inoculation.

(iv) Titration for haemolysin activity. The haemolytic activity of the material prepared in (i), (ii) and (iii) above, was titrated by making serial doubling dilutions in 0.5 ml volumes of phosphate buffered saline at pH 7.3 in World Health Organisation (W.H.O.) haemagglutination plates. An equal volume of 1% suspension of horse erythrocytes (twice washed by low-speed centrifugation and resuspended in phosphate buffered saline) was added to each well in the series. A negative control was set up using the diluent and erythrocyte suspension. The erythrocyte suspension

was mixed with the test material in each well with a pipette and the plate was incubated at 37°C for 2 hours before reading the results. The degree of haemolysis at any dilution was scored to indicate complete (+++), moderate (++) or weak (+).

(v) Toxicity of culture preparations. The toxicity of preparations was tested in vivo by inoculation into mice and rabbits.

Mice: a dose of 1 ml of each preparation examined was injected intraperitoneally into a few mice aged approximately 6 weeks. The animals were observed for 1 week to record illness or deaths.

Rabbits: heated broth culture supernates from 6-day cultures of 4 strains were injected intradermally into the skin over the back of a rabbit previously prepared by shaving. Injections were made using tuberculin-type disposable syringes and doses of 0.2 ml were placed 2 cm apart. A control injection of 0.2 ml of tryptone soya broth was also made. The sites of injection were examined for reactions at 3 hours, 6 hours, 18 hours and then at intervals of 24 hours for 3 days.

(p) Viable cell counts

Viable counts to determine the growth phases and to determine cell-populations in inocula were carried out by the surface viable-count technique of Miles and Misra (1938) as outlined by Cruickshank (1965, p.870). Only 3 strains were examined to obtain information about

the approximate bacterial numbers contained in inocula used in antimicrobial sensitivity tests and in bacteriophage and bacteriocin sensitivity tests.

Some counts were also carried out by the "surface spreading" technique (Cruickshank, 1965, p.870) to determine ultraviolet (UV) inactivation of cultures and to determine the proportions of colony dissociants in nutrient broth cultures of strains of Ps.pseudomallei.

(q) Resistance to heat at 56°C for 1 hour

1 ml volumes of 18-hour nutrient broth cultures of all strains were pipetted into pre-warmed 3" x $\frac{3}{8}$" glass test-tubes and were placed in a waterbath at 56°C for 1 hour. A standard loopful of each culture after treatment was inoculated into a 10 ml volume of nutrient broth which was incubated at 37°C for 72 hours. The cultures were examined daily for growth.

(r) Resistance to ultraviolet (UV) irradiation

Five strains (4 lysogenic and 1 non-lysogenic) were used in this study. A 30 ml volume of nutrient broth was inoculated with 0.3 ml of an overnight nutrient broth culture and was incubated for 6 hours at 37°C in a shaking waterbath[1] (80 strokes/minute). The viable count of this 6-hour culture was determined by the "surface-spreading" method and immediately afterwards the culture was dispensed in 2 ml amounts into 14 small sterile petri-plates (5 cm diam.). The plates were exposed in a covered Cabinet[2] to the radiation from

[1] Grant Instruments Ltd., U.K.
[2] "Luckham" Inoculating Cabinet

a Phi l ips 15 watt germicidal lamp placed 40 cm above them. The dishes containing the suspensions were rocked by hand occasionally and at 1 minute intervals for the first 10 minutes and at 5 minute intervals thereafter a plate was removed from the cabinet to an area of subdued lighting. Serial ten-fold dilutions in nutrient broth were immediately prepared using a "Rotamixer" to ensure thorough, rapid mixing and counts made by the 'spreading' technique. The plates were then incubated for 48 to 72 hours at 37°C in an unlit incubator before counting to estimate the surviving fractions.

(s) <u>In vitro sensitivity to antimicrobial chemotherapeutic agents</u>

(i) Disc-diffusion test. Sixty-one primary strains (Appendix B) were tested against 16 antimicrobial agents (including 14 antibiotics) using sets of Oxoid"Multodisks". In addition, 4 strains of <u>Ps.aeruginosa</u> were included in the study. Initially nutrient agar (Oxoid) was used as the test medium but subsequently all tests were done on Oxoid "Diagnostic Sensitivity Test Agar", with or without 7% defibrinated horse blood (Oxoid). The plates were poured to a standard thickness using 15 ml volumes per standard disposable petri dish (9 cm diam.). The test strain was prepared by inoculating a 10 ml volume of nutrient broth from a slope culture. The broth culture was incubated for 24 hours and a standard loopful then inoculated into a fresh 10 ml volume of nutrient

broth which was incubated for 18 hours and then diluted 1 in 100 in nutrient broth for seeding the test plates. The plates were surface-dried for about 30 minutes and each plate was flooded with an approximately 3 ml volume of the diluted culture. The excess fluid was carefully drained with a pasteur-pipette and plates were left to stand on the bench for about 10 minutes before placing the 'Multodisks'. After a further 15 to 20 minutes standing on the bench (to allow for diffusion of antibiotics) the plates were placed in the incubator. The diameter of the zones of inhibition were measured after 24 hours' incubation.

(ii) Tube dilution test to determine the minimum inhibitory concentrations of antibiotics (MIC). The MIC was determined by a two-fold dilution procedure (Cruickshank, 1965, p.899) on 24 strains (Appendix B). The dilutions of antibiotics were prepared in tryptone soya broth in bulk (50 ml volumes) and ranged from 100 µg/ml down to 0.78 µg/ml. A 2 ml volume of each dilution was dispensed into a series of tubes (3" x $\frac{5}{8}$") and a 2 ml volume of tryptone soya broth containing no antimicrobial agent was placed in a 'control' tube in the series. A 0.5 ml volume of a 1 in 10^2 dilution of an 18-hour tryptone soya broth culture was then added to each tube so that the final dilution of the antibiotic ranged from 80 µg/ml down to 0.625 µg/ml. The number of viable cells in the inoculum was estimated for a few strains selected at random by colony counts using the technique of Miles and Misra (Cruickshank, 1965,

p.870). The tubes were incubated for 18-20 hours before reading the MIC - the highest dilution showing no visible turbidity.

3. RESULTS

(a) Motility

All strains were motile.

(b) Morphological characters and staining reactions

All strains were Gram-negative and non-acid fast. The morphological forms seen included bacillary and coccobacillary forms. The coccobacillary forms predominated in the young "smooth" (S) type cultures and were also discernible in the "mucoid" (M) type cultures. The bacillary forms predominated in the other culture types - the "rough" (R), the "parchment-like" (Rr) and the"rugose" (SR). The characters of these colony types have been described elsewhere (see p. 90).

Bipolar staining (Plates 1 and 2) was most evident in the "R", "SR" and the "Rr" type cultures whether they originated from recently isolated strains or from old laboratory stock cultures. Bipolar stained cells were also encountered in preparations of the 'S' type and the 'SR' type cultures although in much smaller proportions. The "M" type cultures stained weakly and therefore the bipolar staining property was not evident.

Occasionally, long rods (short filaments) two to three times the size of the normal bacillary forms were seen in stained preparations examined under the light microscope and these appeared to correspond to the elongated cells seen in the electron microscope (Plate 3). Preparations examined under the light

microscope of cultures of the "R", "Rr", "SR" and "S" forms grown on 2% sodium chloride agar showed abundant, faintly stained, sinuous forms (Plate 2) in addition to curved rods. These sinuous forms appeared to be non-septate. Such preparations examined in the electron microscope did not reveal sinuous filaments but showed chains of bacilli and coccobacilli (Plate 4).

In all the strains examined it was found that flagella arose from one pole (Plate 5) except in cells that were about to divide. The cells were multitrichous (2 to 6 flagella) although among such multitrichous cells could be seen numerous cells without flagella and the occasional cell carrying a single flagellum. Detached flagella and fragmented flagella were also seen.

Appendages similar to fimbriae (pili) described for some species of the Enterobacteriaceae were seen (Plate 6) but it often necessitated the examination of many cells on the electron microscope grid to detect only a few cells carrying such appendages. Often, strains that yielded fimbriated cells did not do so consistently but only intermittently over several subcultures. Moreover, fimbriated cells were more often found in preparations from young cultures grown on nutrient agar than in nutrient broth. Preparations from some strains irrespective of their colony type showed no appendages similar to fimbriae.

Capsules and pseudo-capsules were not seen in specimens studied either by electron microscope or by the India-ink method.

(c) Cultural characters

(i) Colony characters of 'primary' cultures: The preparations of 'primary' cultures (lyophilised stock cultures) from the original strains received from the various sources was from single colonies on nutrient agar plates incubated for 24 hours. At this stage of incubation, colony dissociation was not noticeable and the colonies of each culture appeared to be homogeneous, either smooth or more rarely rough in surface texture. The smooth colonies were circumscribed, 1.0 to 1.5 mm diameter, translucent to opaque, convex or slightly umbonate with margins regular or slightly crenated. The colonies with the rough surface were rather smaller than the smooth colonies first described and were dull and wrinkled, the wrinkled area tending to become heaped up at the centre with the periphery remaining flat and the edge tending to be thickened and lobate. These colonies were found to be markedly tenacious so that a whole colony could be picked from the surface of the medium by touching with a bacteriological loop. On the basis of these colonial appearances, the lyophilised single colony cultures representative of 116 of the 118 original strains were considered as belonging to two groups - smooth or rough - and were lyophilised. Two of the 118 original strains were found to be non-viable. The lyophilised cultures will be referred to as the 'primary' cultures.

(ii) Cultural variation: When these 'primary'

(lyophilised) cultures were reconstituted and incubated for 24 - 36 hours, they gave rise to colonies of the type used to prepare the lyophilised culture but on further incubation (48 - 72 hours), these colonies developed further and differentiated into one or more colonial forms. These colonial variations were found largely in those cultures that were initially considered as of a smooth variety. Some of these smooth cultures yielded as many as 4 different colony forms per culture. The cultures initially considered as of a rough texture were not found to dissociate freely and only one of these showed some colonies other than that of the rough type. These aberrant colonies found on subculture of 'primary' strains were at first considered as contaminants but apart from their differences in colony morphology they were found to resemble the 'primary' strains in cultural, biochemical properties, motility test and in the reaction to the Gram stain. That they were dissociants of the 'primary' cultures became apparent on subculture of these aberrant forms. They in turn showed dissociative tendencies, some of them giving rise to colonies of the 'primary' type cultures - the smooth or rough textured Ps.pseudomallei cultures.

Table 1 shows the distribution of colonial types on nutrient agar plates incubated for 48 to 72 hours. Only 27 (23.3%) 'primary' cultures were found to yield colonies that were uniformly "smooth" (S), circumscribed, 2-3 mm diameter, convex, translucent, whitish or

TABLE 1

Distribution of colonial types in 'primary' cultures (lyophilised cultures) of 116 strains

Colony type	Total number of cultures	Percent cultures
"S" colonies only	27	23.3
"S" colonies as predominant type with some "M" colonies	15	12.9
"S" colonies as predominant type with some "R" and "Rr" colonies	3	2.5
"S" colonies as predominant type with some "SR" colonies only	39	33.6
"SR" colonies as predominant type with some "S" colonies	16	13.8
"SR" colonies as predominant type with some "S" and "M" colonies	8	6.9
"SR" colonies as predominant type with some "S", "M" and "dwarf" rough colonies	1	0.9
"R" colonies only	6	5.2
"R" colonies as predominant type with some "S" colonies	1	0.9

yellowish in colour, non-tenacious and easily emulsifiable in broth or saline. These plate cultures showed no metallic sheen and were devoid of any aromatic odour. On further incubation or on storage at room temperature, the colonies grew to a size of 4-5 mm diameter and turned uniformly yellowish in colour. Another 15 (12.9%) 'primary' cultures, all from the smooth group yielded "S" colonies as the predominant variety on the plates with "mucoid" (M) colonies occurring among them either as discrete colonies or as a satellite or sector of an "S" colony (Plate 7). These "M" colonies in the initial stages of growth were similar in size or larger than the "S" colonies on the same plate but were distinguishable from them by their larger size or darker shades of colour, by their glistening appearance and by their marked adherence to the surface of the medium. As incubation progressed, they tended to grow larger (approximately 3 - 3.5 mm diameter in 72 hours) than the "S" colonies and often turned to a tan or brown colour. These colonies were less emulsifiable in broth or saline, usually suspending as a ropy mass. Subculture of the "M" colonies to nutrient agar plates showed that, like the "S" colonies they lacked any aromatic odour or a metallic sheen. They retained their adherence to the surface of the medium but unlike the mucoid colonies described for some other bacteria, they were not found to flow on the medium.

Smaller numbers of 'primary' cultures originally placed in the smooth group though showing predominantly "S" type colonies also gave rise to some colonies either of the "rough" (R) type similar to, if not identical, with those in cultures placed in the rough group or of a type similar to the "R" type but very flat, parchment-like and less wrinkled, with the wrinkles spreading radially from the centre to a thickened edge. These in comparison with "S" type colonies on the same plate, were much larger (Plate 8). For purposes of description, these parchment-like colonies have been designated the "Rr" type.

Of the 'primary' cultures which had been placed in the smooth group, 55.2% were found to yield colonies having some of the characters of the "S" type and some of the "R" type. These will be designated the "SR" type colonies. They occurred either as sole colonial type in each culture or as the predominant variety often associated with "S" or sometimes with the "M" types (Plate 11b) and, in a few strains, all 3 types were found present. In one culture, the "SR" type colonies were found as the predominant variety with a few colonies of the "S", "M" and a very small, rough, yellow, "dwarf" colony. The "SR" colonies were found to be no different from the "S" colonies on 24-hour plate cultures but in 36-48 hours (Plate 9), they were found to differentiate into round, opaque colonies, dense, granular and pitted at the centre or sometimes umbonate, the periphery remaining smooth and translucent

As incubation progressed (48-72 hours) they became rugose at the centre leading to corrugation more or less confined to the centre, the periphery becoming lobate but retaining its flat, smooth appearance (Plate 10). The colonies were not tenacious and were easily emulsifiable and their cultures were characterised by a pronounced aromatic (sweet smelling) odour and a distinct metallic sheen which can be best seen in the areas of confluent growth. Continued incubation (72 hours or longer) led to further development, each colony reaching 4-5 mm in diameter, the central region of the colony showing a heaped-up corrugated growth (Plate 11.a-), the periphery retaining its lobate shape and its smooth texture. By 72-96 hours' incubation, the colour characteristics of this colony type also became remarkable. Many of them that were initially dirty white to yellowish were found to turn to a buff, lavender, pinkish (Plate 12.ab) or rarely a purplish pink colouration. Such pigmentation was best seen in areas where the growth was confluent.

Only 1 of 7 primary cultures initially placed in the rough group gave rise to colonies consisting of both "rough" (R) and "smooth" (S) type colonies, the "R" type remaining the predominant variety in this culture (strain 23). The other 6 cultures gave rise exclusively to the "rough" (R) type colonies (Plate 13). These "R" type colonies on continued incubation (48-72 hours) became markedly more corrugated and heaped-up (Plate 14). They attained a size of 3 mm diameter, approximately. Like the "SR" cultures, they yielded

an aromatic odour but the cultures lacked a metallic sheen. The "R" colonies were also distinguishable from the "SR" type by their tenacious character and by their wrinkled surface evident within 24 hours growth. These colonies, unlike the "SR" type, were also found to suspend poorly in either normal saline or in nutrient broth, clumping being observed in both.

Table 2 shows the percentage of dissociants found in periodical platings from nutrient broth cultures started off at different pH levels and inoculated with various colonial types ("S", "SR", "R" and "M"). The "R" type strain has not given rise to dissociants in any of the 3 cultures started off at pH 7.0, 7.4 and 7.8 respectively. The pH of two of the cultures read at day-4 has shown a drop and, in the case of the culture started off at a pH of 7.0, it has shown a shift to acidity. The "M" type cultures also showed no dissociants and in their case, the pH readings taken at day-4 have shown a shift to high alkalinity. The "SR" type strain has given rise to "S" type colonies (Plate 15) regardless of the starting pH. The percentage of the "S" type colonies in "SR" cultures increased as the incubation periods lengthened. A higher percentage of such dissociants occurred in the culture that started off at a pH of 7.8. The pH of these "SR" cultures has shown an increase and has been most marked in the culture started off at pH 7.0.

The "S" type cultures have shown differences in their dissociative tendencies. Strain 108-S has given rise

TABLE 2

Percentage of dissociants on plates from broth cultures started off at different pH levels and incubated for varying periods

Culture No.	Colony type of culture	Starting pH of culture	Percentage of dissociant colonies at				pH at day-4
			Day-1	Day-2	Day-3	Day-4	
108	"S"	7.0	3%("SR")	2%("SR")	0	0	7.4
48	"S"	"	0	0	5%("M")	42%("M")	8.2
4	"SR"	"	0	3%("S")	3%("S")	12%("S")	7.9
61	"R"	"	0	0	0	0	6.8
15	"M"	"	0	0	0	0	8.4
108	"S"	7.4	0	0	0	0	7.9
48	"S"	"	0	0	1%("M")	12%("M")	8.2
4	"SR"	"	0	0	1%("S")	10%("S")	7.8
61	"R"	"	0	0	0	0	7.4
15	"M"	"	0	0	0	0	8.3
108	"S"	7.8	0	0	0	0	8.2
48	"S"	"	0	0	6%("M")	38%("M")	8.2
4	"SR"	"	0	0	6%("S")	18%("S")	7.9
61	"R"	"	0	0	0	0	7.6
15	"M"	"	0	0	0	0	8.4

to a few "SR" type colonies at day-1 to day-2 in the culture that started off at pH 7.0 but has shown no dissociants thereafter nor has it given rise to any dissociants in the cultures started off at pH 7.4 and 7.8. On the other hand, the strain 48 has given rise to a high percentage of "M" type colonies particularly as the cultures aged - day-4 cultures. The pH readings at day-4 have shown a shift to high alkalinity (pH 8.2) even although the cultures were started off at different pH levels. Strain 108-S has also shown increases in pH by day-4, but the shifts have not been as marked as with strain 48-S.

Most of these results point to a probable influence of the changing pH that occur during growth as a factor in the establishment or in the selection and establishment of dissociant types.

The results (Table 3) of the serial transfer of 16 selected colonies (including "M" and "Rr" colonies) picked from plates of 9 'primary' cultures showed that the "R" and the "Rr" types were more stable. Two of the "R" type colonies derived from "R" type 'primary' cultures did not give rise to any dissociants. The other "R" type (strain 23-R) from a plating from a 'primary' culture in which both "R" and "S" colonies were found, gave rise to dissociants of the "S" type once during the serial transfers. Similarly, the one "Rr" type colony (strain 4-Rr) picked from a plating from a 'primary' culture in which 3 colony types ("SR", "S" and "Rr") were found, was seen to give rise to dissociants

TABLE 3

Re-dissociation of 'secondary' dissociants of Ps.pseudomallei

Primary strains	'Secondary' dissociant	Number of colonial types found by the 10th transfer	
		Number of variant colonies	Colonial types
2 (smooth)	2-S	4	"M"; "SR"
"	2-SR	1	"S"
4 (smooth)	4-S	3	"M"; "SR"
"	4-SR	2	"S"
"	4-Rr	1	"S"
15 (smooth)	15-M	None	
"	15-S	4	"M"
18 (smooth)	18-S	3	"M"
"	18-M	3	"S"
23 (rough)	23-R	1	"S"
"	23-S	3	"SR"
24 (rough)	24-R	None	
48 (smooth)	48-S	5	"M"; "SR"
61 (rough)	61-R	None	
115 (smooth)	115-SR	2	"S"; "M"
"	115-S	3	"SR"; "M"

of the "S" type once. Of the 2 "M" colonies picked from 'primary' cultures giving rise to "S" and "M" colonies one yielded "S" type dissociants during some but not all transfers. The other "M" culture (15-M) did not give rise to any dissociants but the growth on these plates was not profuse and on some plates in the series only a few "M" colonies were found limited to the area of the initial inoculum (Plate 16.a), indicating a loss of viability during transfers. The 7 cultures of the "S" type colonies and the 3 cultures of the "SR" type colonies all yielded dissociants though not at every successive subculture. The dissociants to which they gave rise fell into one or more of the recognisable types ("S" to "SR", "M", "Rr" and "SR"; "SR" to "S" "M" and to a "dwarf" rough type; "M" to "S" etc.). However, identical colonial types from different cultures did not give rise to a similar range of dissociants and they also differed in the frequency with which they gave rise to dissociants. The dissociants arising from the 'secondary' dissociants also showed dissociative tendencies and sometimes re-dissociated (Plates 16b, 16c and 16d).

(iii) Odour: A sweet aromatic, non-ammoniacal odour was clearly detectable in the "SR", "R" and "Rr" type cultures on nutrient agar plates incubated for 48 hours. Such an odour was not detectable in the "S" and the "M" type cultures.

(iv) Metallic sheen: A metallic sheen developed

early in the course of incubation (36-48 hours) in cultures of the "SR" type on nutrient or blood agar plates. It was most noticeable in the areas of confluent growth. The metallic sheen was not detectable on "S", "M", "R" and the "Rr" cultures but after 72 hours' incubation, some of the "S" type cultures developed a sheen but these were mainly among those where the "SR" type dissociants were found occurring.

(v) Pellicle growth on the surface of liquid media: In nutrient broth, the pellicle growth at 24 hours was evident in the "R", "Rr" and the "SR" type cultures. In the "SR" type cultures, the medium below also became turbid. Further incubation resulted in the progressive development of the pellicle (Plate 17-A & B). The "S" and the "M" type cultures showed little tendency to develop pellicles within 48 hours (Plate 17-C). After 48 hours' incubation, some "S" type cultures developed a thin or a hardly discernible pellicle but further incubation (5-7 days) did not lead to progressive thickening of the pellicle, and instead the bacterial growth settled to the bottom of the bottle. On swirling the culture contents, the cell deposits of most of the "S" type cultures usually resuspended uniformly in the medium but a few of the "S" type cultures and all "M" type cultures were seen as ropy masses in the medium.

(vi) Changes in pH in nutrient broth cultures: The

different changes in pH observed in nutrient broth cultures appeared to be associated with specific colonial types (Table 4). The majority of the cultures in which the pellicle growths were abundant ("R", "Rr" and the "SR" types) gave pH values ranging from 6.8 to 7.9 in 4-7 days while many of those that developed little or no pellicle ("S" and "M" type cultures) gave pH values ranging from 7.6 to 8.4. The "M" type cultures consistently produced a high alkalinity - pH 7.9 to 8.4 within 96 hours.

(vii) Growth on Sabouraud's Dextrose Agar: All strains grew well on this medium but "S" cultures were found to dissociate into the parchment-like "Rr" cultures. The "M" type cultures were not found to dissociate.

(viii) Growth on nutrient agar containing different concentrations of sodium chloride: All of the 117 cultures of Ps.pseudomallei grew on 2% sodium chloride but only 14 strains were able to grow on 3% sodium chloride. Those that grew at this concentration showed only a fine growth or very minute colonies within 48 to 72 hours' incubation. None of the strains was found to grow on 4% sodium chloride agar. Six strains of Ps.aeruginosa grew equally well on the media containing 2%, 3% and 4% sodium chloride. Incubation for 1 week at 37°C resulted in colour changes. Sixty-nine cultures of Ps.pseudomallei showed yellow to golden yellow colonies.

TABLE 4

pH changes in nutrient broth cultures

Culture No.	pH at day: 1	2	3	4	5	6	7
5-SR	7.2	nt	nt	7.0	nt	nt	nt
7-S	7.2	nt	nt	6.9	nt	nt	nt
14-M	7.6	7.9	7.8	8.1	7.9	7.9	8.0
15-M	7.1	7.3	7.8	8.4	7.9	8.3	8.3
23-S	7.0	7.6	8.0	7.8	7.9	7.8	7.7
23-SR	7.1	7.1	7.0	7.1	7.3	7.4	7.4
24-R	7.1	7.1	7.0	7.1	7.5	7.5	7.4
27-SR	7.0	6.8	6.6	6.8	6.9	7.4	7.3
30-SR	7.2	7.2	7.1	7.0	7.2	7.2	7.3
33-SR	7.2	7.2	7.6	7.4	7.4	7.3	7.3
42-S	7.4	8.6	8.4	8.1	7.9	8.8	7.8
42-M	7.7	8.4	8.8	8.7	7.9	8.7	7.7
100-S	7.2	7.1	6.7	6.8	6.9	7.1	7.3
100-SR	7.4	7.0	7.2	7.4	7.6	7.2	7.0
108-SR	7.2	nt	nt	7.8	nt	nt	6.8
108-S	7.3	7.4	7.6	7.7	7.4	7.6	7.6

nt = not tested

(ix) Growth on cetyltrimethyl ammonium bromide (CTAB) agar: All primary strains of Ps.pseudomallei and their dissociants failed to grow on this medium. The strains of Ps.aeruginosa tested grew well on this medium. The results are summarised in Table 5.

(x) Growth on MacConkey agar, Desoxycholate citrate agar (DCA) and on Salmonella-Shigella (S-S) agar: All strains grew on MacConkey agar, some giving rise to pink colonies (see Table 5). The ability to grow as pink colonies did not appear to be due to the breakdown of the lactose in the medium since the medium prepared without lactose also showed pink colonies.

The inhibition of growth on S-S agar and on DCA was not complete for, on some occasions during repeat tests, some of the strains showed a few small colonies or a slight but visible growth on the site of the stab-inoculum on S-S agar and DCA plates. The Ps.aeruginosa strains were not inhibited by either medium (see Table 5).

(xi) Growth at different temperatures of incubation: All strains grew at 42°C but growth appeared to be slower than at 37°C. The strains did not grow at 5°C but a hardly discernible growth was evident in some (24 strains) incubated at 21°C. The strains of Ps.aeruginosa did not grow at 5°C but gave turbid growths at 21°C and at 42°C. These results have been included in Table 5.

(xii) Growth under anaerobic conditions: None of the strains was found to grow on nutrient agar or in

Growth of Ps.pseudomallei and Ps.aeruginosa on various media

Medium and conditions of growth	Period of observation (days)	Ps.pseudomallei strains			Ps.aeruginosa	
		Percent.of 116 cultures showing			Percent.of 6 cultures showing	
		Good growth	Partial inhibition	No growth	Good growth	No growth
Nutrient agar (anaerobically)	4	0	0	100	0	100
Glucose broth (anaerobically)	4	0	0	100	0	100
Nitrate broth (anaerobically)	4	100	0	0	100	0
Nutrient agar or broth at 5°C	7	0	0	100	0	100
Nutrient agar or broth at 21°C	7	0	27.1 (fine cols)	72.9	nd.	nd.
Nutrient broth at 42°C	3	100	0	0	100	0
Bile salts (10%) agar	3	100	0	0	100	0
Sabouraud dextrose agar pH 5.2	3	100	0	0	100	0
MacConkey agar ("Oxoid" CM7)	3	100	0	0	100	0
Desoxycholate citrate agar ("Oxoid"CM35)	7	0	69.5	30.5	100	0
Salmonella-Shigella agar ("Oxoid")	7	0	16.1	83.9	100	0
Cetyltrimethyl ammonium bromide (0.1%) agar	7	0	0	100	100	0
Sodium chloride (2%) agar	4	100	0	0	100	0
Sodium chloride (3%) agar	7	0	11.9	91.5	100	0
Sodium chloride (4%) agar	7	0	0	100	100	0

nd.= not done

glucose broth cultures under anaerobic conditions but growth occurred within 48 hours in nitrate broth cultures (see Table 5). The'control'cultures grew well on all media, under aerobic conditions.

(xiii) Pigment production: None of the strains produced any diffusible pigments in nutrient agar although pigmentation of the colonies was frequently seen in ageing cultures. A variety of such colony colourations (white, buff, pale to dark yellow, tan, brown, pinkish to purplish-pink) were seen and some of these colony colourations appeared to characterise the colony type i.e. "M" strains on ageing were found to be of a yellow, tan or brown colour (Plate 11a) and the "SR" types usually developed a pinkish to purplish-pink colour (Plate 12). Repeat cultures did not always give similar results and therefore classifications on the basis of colony colours was not possible. Such colour changes were not evident in the strains of Ps.aeruginosa but 4 of 6 strains studied produced blue-green diffusible pigment within 3 to 4 days of incubation. However, even the chromogenic strains of Ps.aeruginosa were not found to be consistent in their production of this blue-green pigment as seen in the course of sub-cultures from the stock strains for various other experiments. A noticeable feature of the Ps.pseudomallei strains was their ability to develop into bright yellow or orangish colours when grown on 2% sodium chloride agar for 6-7 days at 37°C. Forty percent of the strains,

regardless of colony type, developed such colourations. Ps.aeruginosa strains were not found to develop such colony colourations.

(xiv) Viability of cultures on storage at room temperature or in the cold: Eighty-two percent of the nutrient agar slope cultures stored at room temperature were found to be viable at 4 weeks, 70% remained viable up to 3 months, 58% remained viable up to 6 months, 42% remained viable up to 1 year and 28% remained viable up to 3 years. However, many of the original "SR" type cultures were found to have dissociated into the "S" type. Some of the "M" type cultures (derived from plates of predominantly "S" or "SR" type cultures) were found to be non-viable within 1 to 3 weeks. Other "M" cultures (similarly derived) were found to yield only "S" colonies when they were stored for periods more than 2 to 3 weeks.

On nutrient agar slopes at 5°C, none of the cultures was found to be viable after 5 weeks. Fifty percent of the cultures were found to be non-viable by the third week and by the fourth week, the proportion of non-viable cultures had reached 85%.

(d) Biochemical characters

The results of the biochemical characters other than 'sugar' reactions are shown in Table 6. All strains (including dissociants) possessed the following characters: catalase positive, oxidase positive, reduced

TABLE 6

Biochemical characters of 116 strains of Ps.pseudomallei

Test	Number of strains giving positive readings		
	N.C.T.C. Percentage positive (Total of 10 strains)	Sabah, Malaysia. Percentage positive (Total of 16 strains)	Others Percentage positive (Total of 90 strains)
Catalase	100	100	100
Oxidase	100	100	100
Acid from glucose (Hugh & Leifson's O/F)	100*	100*	100*
Nitrate reduction	100***	100***	100***
Arginine hydrolysis	100	100	100
Gelatin liquefaction	100*	100*	100*
Liquefaction of Loeffler's serum	60	75	80.5
Methylene blue reduction	90	100	62.2
Production of H_2S	100	100	92.4
Acid in litmus milk and clot formation	100	100	82.9
Lecithinase activity	100	100	100
Starch hydrolysis	0	0	0
Urea hydrolysis	0	0	0
Indole production	0	0	0
Methyl-red	0	0	0
Voges-Proskauer	0	0	0
Citrate utilisation	100	100	100
KCN resistance	100	100	100
Malonate utilisation	0	0	9.8**
Potassium gluconate oxidation	0	0	0

* some react slowly and not evident within 4 days

** some weak reactions

*** sometimes to nitrogen

nitrates, produced ammonia from arginine, liquefied gelatin within 3 to 14 days, grew in Koser's citrate medium and in KCN broth, failed to hydrolyse urea (except for a very faint pink colouration seen in some strains in 2 to 4 weeks), failed to hydrolyse starch, oxidise gluconate or produce indole and were M.R. negative and V-P negative. All strains also produced lecithinase but in some strains irrespective of the date of isolation, the reactions were weak and delayed (4-7 days).

The majority of the strains produced hydrogen sulphide, produced acid in litmus milk with slow digestion of the clot, reduced methylene blue and caused liquefaction of Loeffler's serum (within 3 to 14 days). Only a small percentage of the strains utilised malonate.

All strains oxidised glucose when tested by the Hugh & Leifson's method and also produced acid without gas when tested in glucose peptone water medium but many gave weak and delayed (5 to 7 days) reactions. The reactions in peptone water media containing other sugars were negligible. A few strains showed a slight acidity as indicated by a yellowish tint in lactose, galactose, trehalose and some of the other sugars but these were often not repeatable. These reactions were therefore interpreted as negative.

(e) Haemolysin production and haemolysin activity of culture preparations

(i) Haemolysis on plates. Haemolysis on sheep or horse blood agar plates incubated at 37°C was not observed

within 48 hours' incubation but in 72-96 hours'incubation, haemolysis was seen in some cultures in the areas of confluent growth. On further incubation or storage at room temperature, the haemolysis became more marked and was similar in appearance to the haemolysis seen with haemolytic strains of Escherichia coli. Furthermore, haemolytic zones became apparent around most if not all colonies in areas where the colony population on a plate was dense. No particular colony types could be associated with the haemolysis but on one occasion, because of the occurrence of predominantly non-haemolytic colonies in the crowded areas of the plate, it was possible to see that the haemolysis was associated with a rough, yellowish, "dwarf" colony type. Colonies growing in isolation in less crowded areas of the plates were never found to be of the "dwarf" type and, the colonies though much larger than those on crowded areas, were not found to show haemolysis though continued storage at room temperature caused some of these well-isolated colonies to produce degrees of haemolysis. Some cultures often those of the "M" or "S" were not found to produce any haemolysis even after prolonged incubation or on storage at room temperature for up to 10 days. This was a feature not only in old laboratory stock cultures but also in some of the more recently isolated strains.

The degree of haemolysis on blood agar appeared to be influenced by the species of red blood corpuscles incorporated in the base medium. Plate 18

illustratesthe differences in the degree of haemolysis by 5 strains of Ps.pseudomallei "replicated" (by stab-inoculation) to sheep blood agar plates prepared the same day from the same batch of base medium and incubated for the same period (96 hours). The haemolysis is most marked on the plate containing horse blood. Plate 19 illustrates the haemolysis caused by 4 strains of Ps.aeruginosa and one strain of Ps.pseudomallei similarly inoculated. These show that Ps.aeruginosa is haemolytic on both horse blood agar and sheep blood agar but the activity is more marked on sheep blood.

(ii) Haemolytic activity of culture preparations. The results of the haemolytic activity of the various culture preparations are shown alongside the results of their toxicity in Tables 7 and 8. The supernates from 3-day broth cultures and from washings from 3-day growths on cellophane-laid medium showed no haemolytic activity. The supernates from many of the 6-day cultures showed degrees of haemolytic activity which was more marked in those from growths on cellophane-laid medium. Even in these, complete haemolysis was usually found to occur in wells containing the undiluted material. The undiluted broth culture supernates caused only a moderate to weak haemolysis. Heating to 100°C for 15 minutes caused an appreciable loss of the haemolytic activity.

Cell extracts obtained by freezing and thawing cell harvests from 3-day tryptone soya agar cultures showed a haemolytic activity comparable with and sometimes

TABLE 7

Haemolysin activity and toxicity in mice of culture preparations from 9 strains of Ps.pseudomallei

Strain and colony type	Preparation	Haemolysin activity — Dilution of material					Toxicity to mice	
		Undil.	1/2	1/4	1/8	1/16	Number ill within 18 hrs.	Number dead within 3*days
4-SR	3.BS.u	-	-	-	-	-	nt	nt
	3.BS.h	-	-	-	-	-	0/4	0/4
	6.BS.u	++	-	-	-	-	nt	nt
	6.BS.h	++	-	-	-	-	4/4	0/4
	3.CS.u	-	-	-	-	-	nt	nt
	3.CS.h	-	-	-	-	-	0/4	0/4
	6.CS.u	+++	++	+	-	-	nt	nt
	6.CS.h	+++	++	-	-	-	4/4	1/4
15-M	3.BS.u	-	-	-	-	-	nt	nt
	3.BS.h	-	-	-	-	-	0/4	0/4
	6.BS.u	-	-	-	-	-	nt	nt
	6.BS.h	-	-	-	-	-	0/4	0/4
	3.CS.u	-	-	-	-	-	nt	nt
	3.CS.h	-	-	-	-	-	0/4	0/4
	6.CS.u	-	-	-	-	-	nt	nt
	6.CS.h	-	-	-	-	-	0/4	0/4
32-R	3.BS.u	-	-	-	-	-	nt	nt
	3.BS.h	-	-	-	-	-	0/4	0/4
	6.BS.u	++	-	-	-	-	nt	nt
	6.BS.h	++	-	-	-	-	4/4	0/4
	3.CS.u	-	-	-	-	-	nt	nt
	3.CS.h	-	-	-	-	-	0/4	0/4
	6.CS.u	+++	++	-	-	-	nt	nt
	6.CS.h	+++	+	-	-	-	4/4	1/4
33-SR	3.BS.u	-	-	-	-	-	nt	nt
	3.BS.h	-	-	-	-	-	0/4	0/4
	6.BS.u	++	-	-	-	-	nt	nt
	6.BS.h	++	-	-	-	-	3/4	0/4
	3.CS.u	-	-	-	-	-	nt	nt
	3.CS.h	-	-	-	-	-	0/4	0/4
	6.CS.u	+++	+	-	-	-	nt	nt
	6.CS.h	+++	-	-	-	-	2/4	2/4
42-S	3.BS.u	-	-	-	-	-	nt	nt
	3.BS.h	-	-	-	-	-	nt	nt
	6.BS.u	++	-	-	-	-	nt	nt
	6.BS.h	++	-	-	-	-	nt	nt
	3.CS.u	-	-	-	-	-	nt	nt
	3.CS.h	-	-	-	-	-	nt	nt
	6.CS.u	+++	++	-	-	-	nt	nt
	6.CS.h	+++	+	-	-	-	nt	nt
74-S	3.BS.u	-	-	-	-	-	nt	nt
	3.BS.h	-	-	-	-	-	nt	nt
	6.BS.u	-	-	-	-	-	nt	nt
	6.BS.h	-	-	-	-	-	nt	nt
	3.CS.u	-	-	-	-	-	nt	nt
	3.CS.h	-	-	-	-	-	nt	nt
	6.CS.u	+++	++	+	-	-	nt	nt
	6.CS.h	+++	+	-	-	-	nt	nt
108-S	3.BS.u	-	-	-	-	-	nt	nt
	3.BS.h	-	-	-	-	-	0/4	0/4
	6.BS.u	-	-	-	-	-	nt	nt
	6.BS.h	-	-	-	-	-	4/4	2/4
	3.CS.u	-	-	-	-	-	nt	nt
	3.CS.h	-	-	-	-	-	0/4	0/4
	6.CS.u	+	-	-	-	-	nt	nt
	6.CS.h	-	-	-	-	-	4/4	3/4
111-SR	3.BS.u	-	-	-	-	-	nt	nt
	3.BS.h	-	-	-	-	-	nt	nt
	6.BS.u	+++	+++	+	-	-	nt	nt
	6.BS.h	+++	++	-	-	-	nt	nt
	3.CS.u	-	-	-	-	-	nt	nt
	3.CS.h	-	-	-	-	-	nt	nt
	6.CS.u	+++	+++	++	-	-	nt	nt
	6.CS.h	+++	++	-	-	-	nt	nt
118-SR	3.BS.u	-	-	-	-	-	nt	nt
	3.BS.h	-	-	-	-	-	nt	nt
	6.BS.u	+++	+++	++	-	-	nt	nt
	6.BS.h	+++	++	-	-	-	nt	nt
	3.CS.u	+	-	-	-	-	nt	nt
	3.CS.h	-	-	-	-	-	nt	nt
	6.CS.u	+++	+++	++	-	-	nt	nt
	6.CS.h	+++	++	-	-	-	nt	nt

Explanatory:-

3 = 3-day culture
6 = 6-day culture
BS = broth supernate
CS = supernate off washings from the growth on cellophane membrane
+++ = complete haemolysis
++ = moderate haemolysis
+ = weak haemolysis
- = negative

u = unheated
h = heated
nt = not tested
* = no deaths occurred after 3 days

TABLE 8

Haemolysin activity and toxicity in mice of cell extracts prepared from 3-day cultures of 6 strains of Ps.pseudomallei

Strain and colony type	Prepara-tion	Haemolysin activity Dilution of material					Toxicity to mice	
		Undil.	1/2	1/4	1/8	1/16	Number ill within 18 hrs.	Number dead within 3*days
4-SR	u	+++	+++	++	+	-	nt	nt
4-SR	h	+++	+	-	-	-	3/4**	1/4**
15-M	u	++	-	-	-	-	nt	nt
15-M	h	+	-	-	-	-	0/4	0/4
33-SR	u	+++	++	+	-	-	nt	nt
33-SR	h	+	-	-	-	-	0/4	0/4
108-S	u	++	+	-	-	-	nt	nt
108-S	h	+	-	-	-	-	4/4	1/4
111-SR	u	+++	+++	+	+	-	nt	nt
111-SR	h	+++	++	-	-	-	4/4	2/4
118-SR	u	+++	+++	++	+	-	nt	nt
118-SR	h	+++	++	-	-	-	0/4	0/4

Explanatory:-

u = unheated
h = heated
+++ = complete haemolysis
++ = moderate haemolysis
+ = weak haemolysis
- = negative
nt = not tested
* = no deaths occurred after 3 days
** = number sick or dead out of a group of 4 mice

stronger than that seen in supernates of cell washings from 6-day cultures grown on cellophane-laid medium, indicating that the haemolytic activity is due to an intracellular haemolysin. Here again, heating did appear to reduce the activity to some degree.

(f) Toxicity of culture preparations

The broth culture supernates inoculated intra-dermally into a rabbit, did not cause any appreciable skin reactions except for a slight reddening at some of the sites noticed at 1 hour but not at 3 hours. No other reactions were seen throughout the period of observation.

In mice, symptoms of illness were seen within 6 to 18 hours in all groups that received intra-peritoneal injections of heated preparations from 6-day cultures. However, many of the animal recovered within 24 to 36 hours. The mortality in these groups was low, the broth culture supernates showing the least toxicity. The heated supernates from 3-day broth cultures and from washings from growths on cellophane-laid tryptone agar did not cause any deaths nor give rise to symptoms of illness in any groups. However, cell-extracts from 3-day cultures grown on tryptone agar showed toxicity. The mortality figures recorded against these extracts of strains did not differ widely from those recorded for the cell-washings of the same strains grown for 6 days on cellophane-laid tryptose agar. In the absence of toxicity studies using unheated preparations it is

difficult to interpret the relationship between haemolysins and these intracellular toxic products. Whatever this toxic material be, it has been found to be thermostable. The haemolysin on the other hand was found to lose some of its activity through heating.

(g) Resistance to ultraviolet irradiation

The data shown in Table 9 have been plotted (Fig.1). The 5 strains were inactivated in 8 to 15 minutes with 50% of the cells in 4 of the 5 suspensions being inactivated within 3 minutes.

(h) Resistance to heat at 56°C for 1 hour

None of the cultures examined was found to withstand this treatment.

(i) Viable cell counts

The growth of the organism in nutrient broth cultures as could be measured from viable cell counts (Table 10) of 3 strains of Ps.pseudomallei is shown graphically in Fig. 2.

The 18-24 hour cultures on transfer to fresh nutrient broth have shown no significant lag phase and have grown exponentially reaching their maximum counts in 15 to 18 hours. A phase of decline in growth was not apparent during the observation period of 36 hours, the cultures remaining in the stationary phase after reaching the peak logarithmic phase. The bacterial counts at 42 hours and 48 hours were not taken as pellicle growths began

Figure I. UV–inactivation of 5 strains of *Ps. pseudomallei*

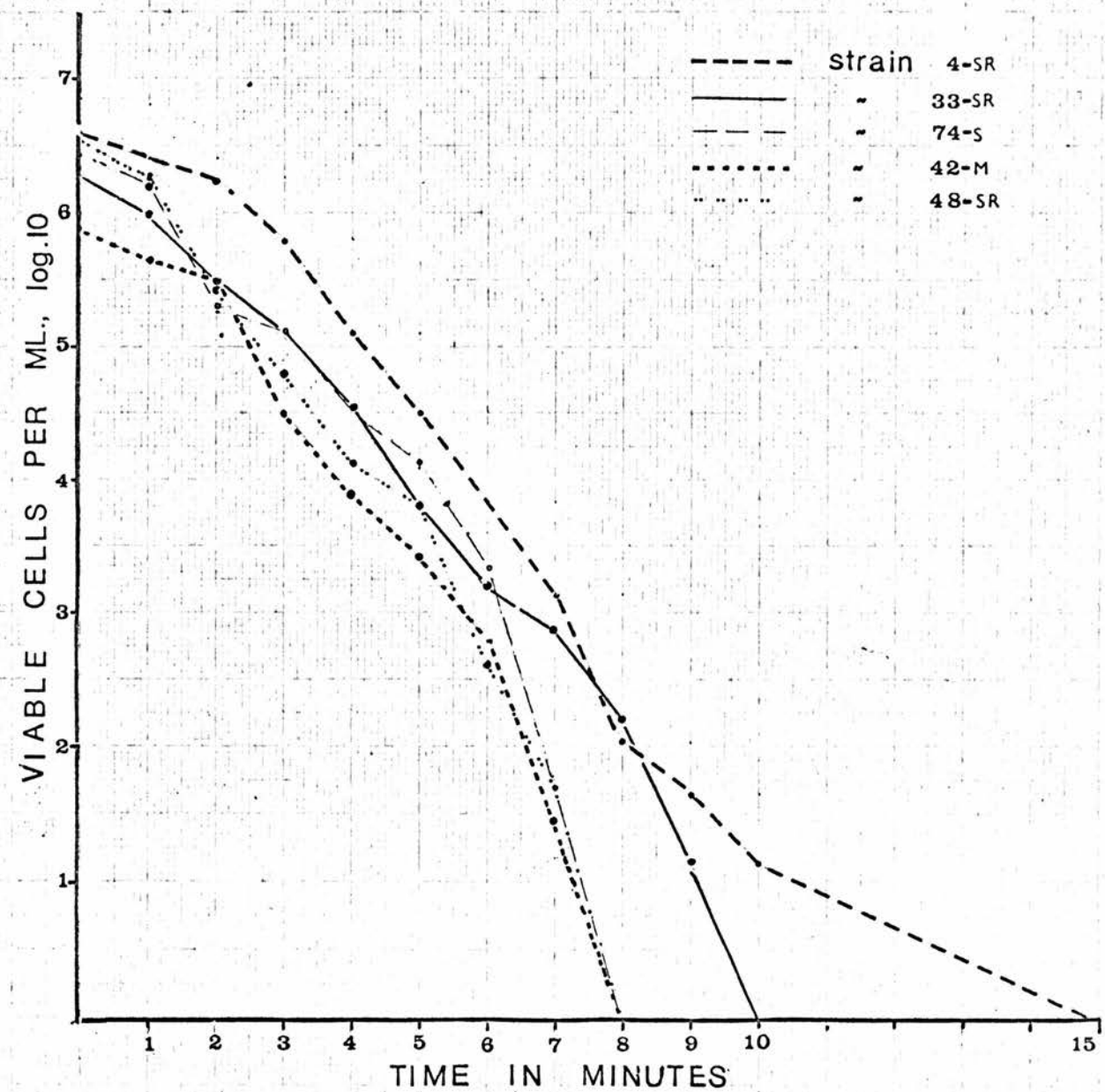

Figure 2. Growth of 3 strains of *Ps. pseudomallei*

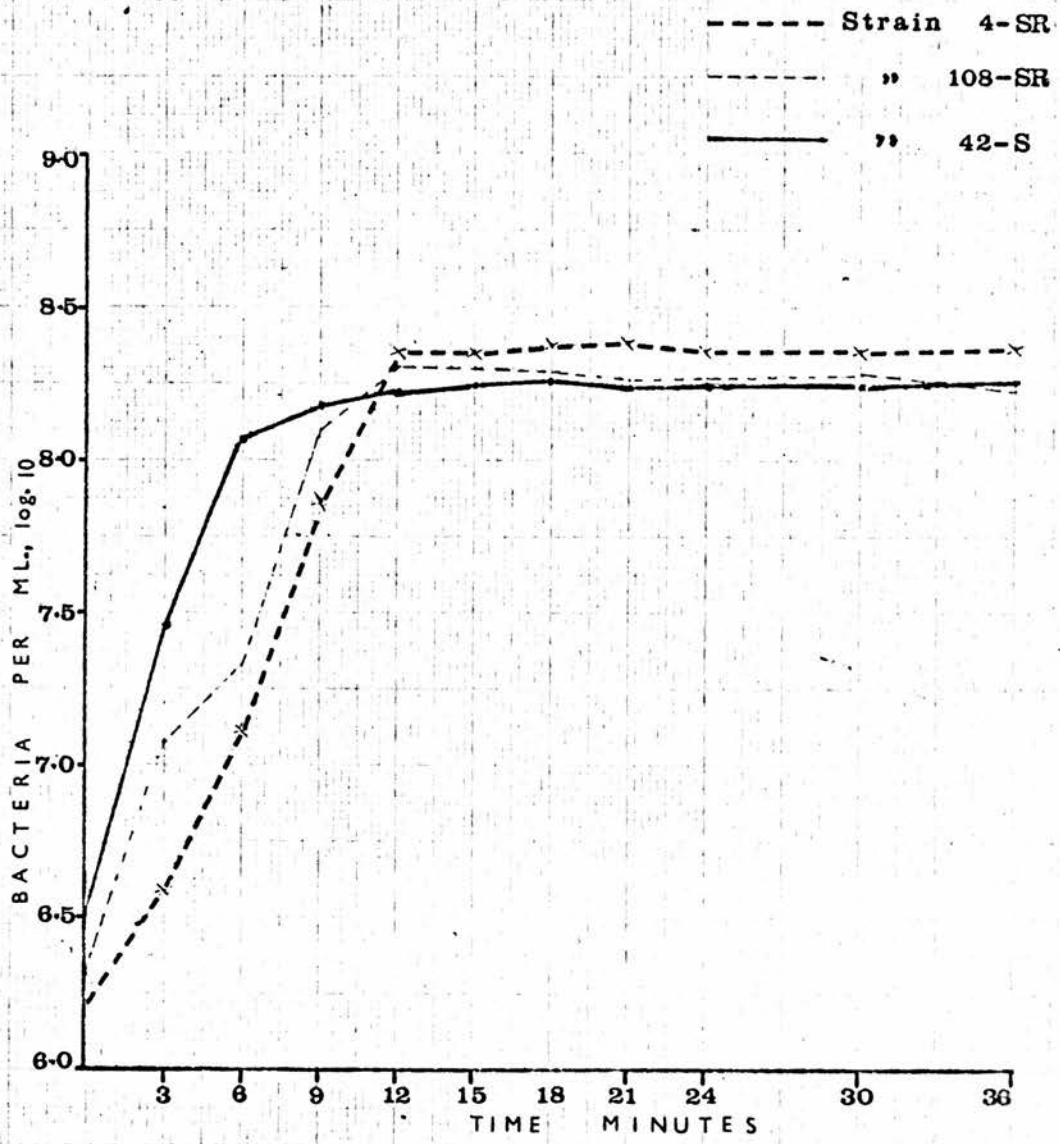

TABLE 9

UV-inactivation of 5 strains of Ps.pseudomallei

Exposure time in minutes	Number of survivors per ml of culture suspension of strain:				
	4-SR	33-SR	48-SR	42-M	74-S
0	5.8×10^6	2.6×10^6	5.6×10^6	8.8×10^5	4.1×10^6
1	4.0×10^6	9.8×10^5	2.6×10^5	6.5×10^5	2.1×10^6
2	2.6×10^6	5.0×10^5	4.2×10^5	5.5×10^5	3.1×10^5
3	5.8×10^5	1.4×10^5	4.8×10^4	4.5×10^4	1.4×10^5
4	1.0×10^5	5.5×10^4	1.2×10^4	8.8×10^3	5.1×10^4
5	4.5×10^4	8.2×10^3	8.0×10^3	4.4×10^3	1.4×10^4
6	8.8×10^3	1.8×10^3	2.6×10^2	7.8×10^2	3.4×10^3
7	1.4×10^3	8.8×10^2	1.7×10^1	1.4×10^1	1.7×10^3
8	0.4×10^2	2.0×10^2	(none)	(none)	(none)
9	6.5×10^1	1.5×10^1			
10	1.8×10^1	(none)			
15	(none)				

TABLE 10

Viable cell counts

Time (in hours)	Bacteria per ml of culture No. -		
	4-SR	42-S	108-SR
0	3.8×10^6	6.2×10^6	6.5×10^6
3	6.6×10^6	1.5×10^7	7.4×10^7
6	2.2×10^7	6.5×10^7	1.5×10^8
9	7.8×10^8	4.0×10^8	3.5×10^8
12	7.0×10^8	6.1×10^8	4.5×10^8
15	7.5×10^8	6.1×10^8	4.9×10^8
18	7.6×10^8	5.8×10^8	5.2×10^8
21	7.5×10^8	5.2×10^8	4.8×10^8
24	7.2×10^8	5.3×10^8	5.0×10^8
30	7.1×10^8	5.5×10^8	4.6×10^8
36	7.4×10^8	5.4×10^8	4.9×10^8

to occur in the "SR" type cultures: even the "S" culture showed some clumping.

There appeared to be a significant difference in the counts obtained at the peak of the logarithmic phase in that strain 4 (subsequently found to be non-lysogenic) showed a count of 7.6×10^8 cells per ml while the other strains (subsequently found to be lysogenic) yielded relatively lower counts.

(j) In vitro sensitivity to antimicrobial agents

(1) Disc diffusion: The data obtained for Ps.pseudomallei and for Ps.aeruginosa are summarised in Tables 11 and 12 respectively. With few exceptions (strains 15 and 113), the strains of Ps.pseudomallei differed little from each other in their sensitivity or resistance to a number of antimicrobial agents and showed patterns distinct from those observed for strains of Ps.aeruginosa, which were invariably found to be sensitive to polymixin, colistin, gentamycin and to a considerable extent to streptomycin (Plates 20a, 20b and 20c).

Strains 15 and 113 differed markedly from all other strains of Ps.pseudomallei in showing a sensitivity to streptomycin and a proportionately greater sensitivity to neomycin (Compare Plates 21a,b with 21c,d). The two strains also differed from the other strains in showing a high degree of sensitivity to gentamycin (Compare Plates 22a, with Plates 22b) and a degree of sensitivity to erythromycin (Plate 24). Strain 15

TABLE 11

Sensitivity of 61 strains of Ps.pseudomallei to 16 antimicrobial agents by the disc-diffusion test

Antimicrobial agent (disc concentration)	Number (Percentage) of strains showing inhibitory zones* with diameter of:			
	>20 mm	16-20 mm	12-15 mm	<12 mm or no inhibition
Chloramphenicol (10 µg.)	25 (41.0)	21 (34.4)	2 (3.3)	13 (21.3)
Erythromycin (10 µg.)	1 (1.64)	0 (0)	1 (1.64)	59 (96.7)
Penicillin G (1.5 units)	0 (0)	0 (0)	0 (0)	61 (100.0)
Streptomycin (10 µg.)	2 (3.3)	0 (0)	0 (0)	59 (96.7)
Tetracycline (10 µg.)	24 (39.3)	22 (36.1)	5 (8.2)	10 (16.4)
Tetracycline (50 µg.)	61 (100.0)	0 (0)	0 (0)	0 (0)
Bacitracin (5 units)	0 (0)	0 (0)	0 (0)	61 (100.0)
Nitrofurantoin (200 µg.)	0 (0)	0 (0)	0 (0)	61 (100.0)
Neomycin (10 µg.)	2 (3.3)	12 (19.7)	35 (57.3)	12 (19.7)
Polymixin B (300 units)	0 (0)	0 (0)	0 (0)	61 (100.0)
Gentamycin (10 µg.)	2 (3.3)	0 (0)	18 (29.5)	41 (67.2)
Cephaloridine (25 µg.)	0 (0)	0 (0)	0 (0)	61 (100.0)
Colistin Methane Sulphonate (200 µg.)	0 (0)	1 (1.6)	0 (0)	60 (98.4)
Carbenicillin (100 µg.)	30 (49.2)	10 (16.4)	16 (26.2)	5 (8.2)
Ampicillin (25 µg.)	19 (31.2)	16 (26.2)	21 (34.4)	5 (8.2)
Sulphamethoxazole/Trimethoprim (25 µg.)	54 (88.5)	1 (1.64)	1 (1.64)	5 (8.2)
Sulphafurazole (100 µg.)	36 (59.0)	6 (9.8)	2 (3.3)	17 (27.9)
Sulphafurazole (500 µg.)	61 (100.0)	0 (0)	0 (0)	0 (0)

*The zones of inhibition were quite clear-cut with all antimicrobial agents with the exception of Sulphafurazole and Sulphamethoxazole/Trimethoprim with which partial inhibition was observed and a hazy growth seen in the area of inhibition.

TABLE 12

Sensitivity of 4 strains of Ps.aeruginosa to 16 antimicrobial agents by the disc-diffusion test

Antimicrobial agent (disc concentration)	Number of strains showing inhibitory zones* with diameter of			
	>20 mm	16-20 mm	12-15 mm	<12 mm
Chloramphenicol (10 µg.)	0	2	1	1
Erythromycin (10 µg.)	0	0	0	4
Penicillin G (1.5 units)	0	0	0	4
Streptomycin (10 µg.)	1	2	0	1
Tetracycline (10 µg.)	0	3	1	0
Tetracycline (50 µg.)	4	0	0	0
Bacitracin (5 units)	0	0	0	4
Nitrofurantoin (200 µg.)	0	0	0	4
Neomycin (10 µg.)	2	2	0	0
Polymixin B (300 units)	4	0	0	0
Gentamycin (10 µg.)	4	0	0	0
Cephaloridine (25 µg.)	0	0	0	4
Colistin Methane Sulphonate (200 µg.)	4	0	0	0
Carbenicillin (100 µg.)	2	0	0	2
Ampicillin (25 µg.)	0	0	0	4
Sulphamethoxazole/ Trimethoprim (25 µg.)	2	0	0	2
Sulphafurazole (100 µg.)	1	0	0	3
Sulphafurazole (500 µg.)	1	0	0	3

was not however identical in its sensitivity pattern to strain 113 for, among other variations, it showed a sensitivity to colistin methane sulphonate (see: Plate 22b).

Although all strains of Ps.pseudomallei showed a high degree of sensitivity to tetracycline at a disc concentration of 50 µg., they showed differences in sensitivity to a lower concentration (10 µg.). Ten strains showed no sensitivity or a zone of sensitivity less than 12 mm diameter. These strains showing resistance to tetracycline were not found to be resistant to chloramphenicol and likewise, the 13 strains showing resistance to the latter agent were not found to be resistant to tetracycline at the disc concentration of 10 µg.

All strains of Ps.pseudomallei were found to be resistant to polymixin B and only a minority of the strains (strains 15, 32, 39, 41 and the strains from Sabah) were found to show degrees of sensitivity to gentamycin. Although the majority of the strains showed large zones of sensitivity against Sulphafurazole and Sulphamethoxazole/Trimethoprim, fine growths were invariably found within such zones. Penicillin had no effect on any strains but the synthetic penicillins acted on a great number of strains.

Different colony types from the same parent strain were usually found to be uniform in their sensitivity or resistance to antimicrobial agents but occasionally a strain was found whose dissociants showed

differences in the degree of sensitivity without alterations in the sensitivity pattern (Plates 23a,b).

(2) Minimum inhibitory concentration (MIC): The MICs of 10 antimicrobial agents to 24 strains of Ps.pseudomallei are shown on Table 13 and the cumulative percent of strains inhibited are shown in Fig.3.

TABLE 13

Minimum inhibitory concentration of 10 antimicrobial agents to 24 strains of Ps.pseudomallei

Antimicrobial agent	Number of strains inhibited by a MIC (µg./ml) of:									Mean MIC
	>80	80	40	20	10	5	2.5	1.25	0.63	(µg/ml)
Chloramphenicol	0	0	0	1	9	13	1	0	0	7.4
Tetracycline	0	0	0	0	0	7	11	2	4	1.81
Kanamycin	0	1	5	16	2	0	0	0	0	32.3
Novobiocin	0	0	0	1	7	15	1	0	0	8.23
Carbenicillin	24	0	0	0	0	0	0	0	0	nw
Gentamycin	10	12	0	0	1	1	0	0	0	nw
Colistin	24	0	0	0	0	0	0	0	0	nw
Neomycin	0	2	14	8	0	0	0	0	0	36.7
Polymixin B	24	0	0	0	0	0	0	0	0	nw
Streptomycin	22	0	2	0	0	0	0	0	0	nw

nw = not worked out

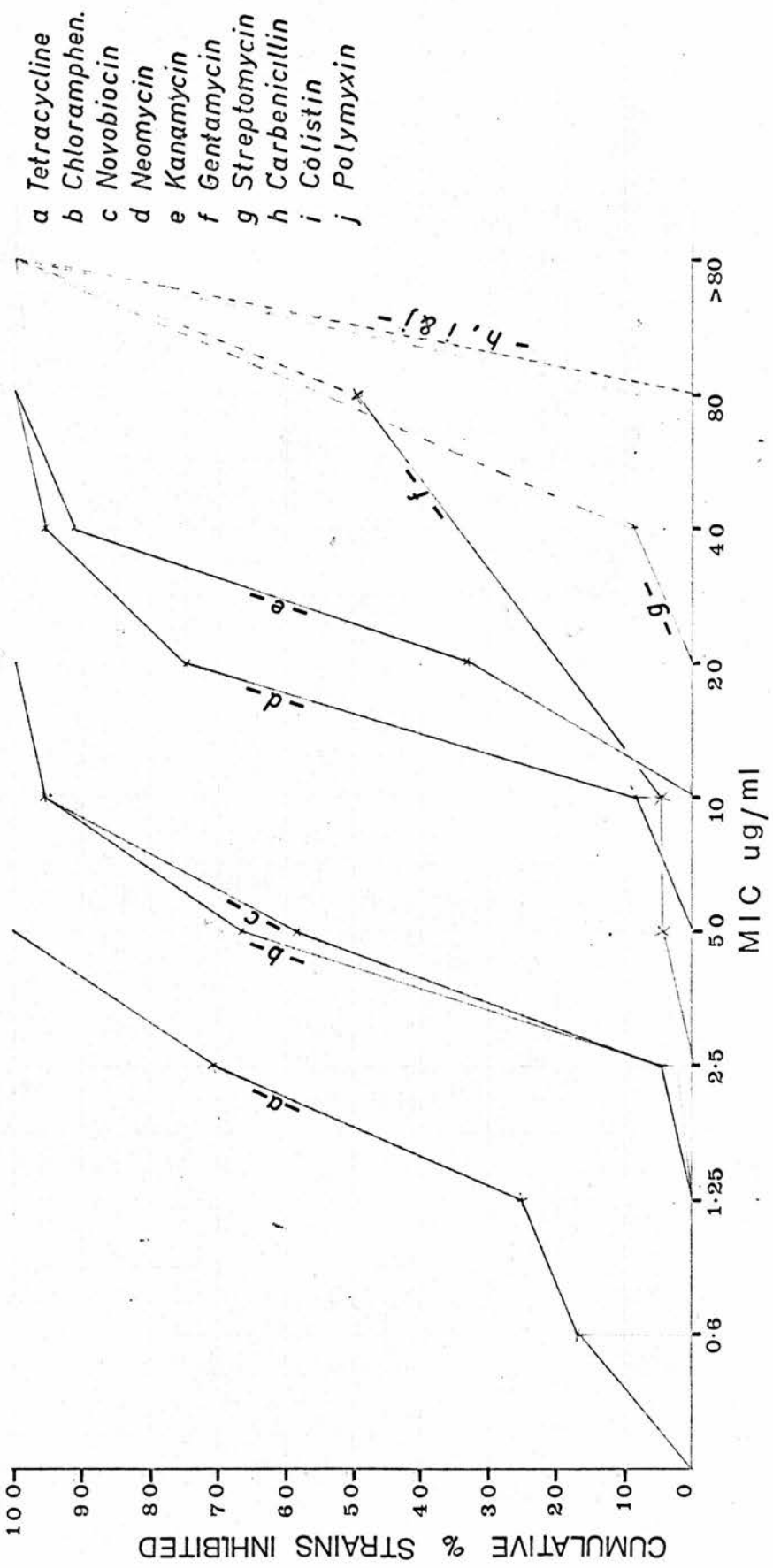

Figure 3. *In vitro* activity of 10 antimicrobial agents against 24 strains of *Ps pseudomallei*

4. DISCUSSION

(a) Morphology and staining

In general, the morphological findings are in agreement with those of previous workers who considered that the usual morphology of Ps.pseudomallei consisted of bacilli and coccobacilli. Whitmore (1912b) and Souchard (1932) also noted the occurrence of sinuous forms in cultures grown in nutrient broth containing high concentrations of sodium chloride and Osteraas et al. (1971) found filamentous Gram-negative rods in peritoneal fluid and tissue sections in a case of human melioidosis. Miller et al. (1948a) encountered filamentous forms in their cultures of Ps.pseudomallei (M.pseudomallei) but these on careful study had appeared to them as long chains of bacilli. The present study has shown that the short filament forms from preparations of nutrient agar or nutrient broth containing sodium chloride correspond to the chains of bacillary and coccobacillary forms seen in the electron microscope. The sinuous forms seen when stained (methylene blue or Leishman's) and examined in the light microscope, were not visible in the electron microscope. This may have been because of the penetration of the negative stain used in electron microscopy.

Nicholls (1930), Finlayson (1944), Cox and Arbogast (1945), Brundage et al. (1968) and Greenawald et al. (1969), using various stains (Gram's, Leishman's or Wayson's), found that bipolar staining was a character

of young cultures of their 'rough' forms but not of their 'smooth' forms. Beamer et al. (1954) found their 'S' and 'R' strains giving poor bi-polar staining reactions. The present findings have shown that a proportion of cells whether from young or ageing cultures show bipolar staining and that such cells can also be found in preparations from 'smooth' (S-type) cultures. An absence of bipolar staining was noticeable only in "mucoid" (M) type cultures but smears from such cultures stained poorly with all the stains used, many cells appearing as shadow forms embedded in a stained matrix possibly made up of extra-cellular slime. Nicholls (1930), Finlayson (1944) and Beamer et al. (1954) observed that their 'smooth' types also showed shadow forms and it is therefore possible that their 'smooth' cultures were a mixture of dissociants consisting of 'smooth' (S) and 'mucoid' (M) cells. 'Mucoid' colonies in addition to the normal 'smooth' and 'rough' colonies were isolated from the blood of several cases of melioidosis (LeGac, Cournes and Bres, 1954). Salisbury and Likos (1970) described organisms from a "smooth mucoid colony type" which stained poorly with Gram's stain with an absence of typical bipolarity. These findings taken together would suggest that the bipolar staining property of Ps.pseudomallei, considered by many workers (including Laws, 1964) to be a uniform characteristic in fresh isolates, may not be a reliable criterion in the identification of the organism.

Finlayson (1944) detected well-capsulated organisms

in smears prepared from pleural and peritoneal fluids of animals experimentally infected with Ps.pseudomallei (B.whitmori). However, he failed to detect any capsulated organisms from smears prepared from primary cultures of materials from infected animals. Fournier and Chambon (1958) observed pseudo-capsules and, in studies on the antigenic components (Chambon and Fournier, 1956b; Chambon, Staub and Bourdet, 1957), they concluded that K antigens were present. Gutner and Fisher (1948) also observed capsulated organisms in smears from exudates of infected guinea-pigs. The present investigation failed to demonstrate any form of capsulation either by the India-ink method or using the electron microscope. This finding is in accordance with the majority of workers who found no evidence for capsulation. However, the strains used in this work had been subcultured several times at least since their original isolation from pathological material. Although the property to form capsules may be lost during culture in artificial media, Finlayson's observation would suggest that the loss is complete on the very first isolation in artificial media. It is therefore questionable whether the capsulated organisms seen in pleural and peritoneal fluids of dying and dead carcases were those of Ps.pseudomallei or those of secondary invaders.

The motility of Ps.pseudomallei has remained one of the principal criteria by which it has been differentiated from the organism of glanders. The number of flagella and their mode of implantation has become

of taxonomic importance and indeed its type of flagellation pointed towards the inclusion of the organism in the Pseudomonadaceae. Nevertheless, the reports on the numbers of flagella and their mode of implantation have not been in complete agreement. Souchard (1932), Legroux and Genevray (1933) and Vaucel (1937) using ordinary light microscopy observed peritrichous flagellation with numbers ranging from 8-20 flagella. Brindle and Cowan (1951) using similar equipment and Kirkpatrick's stain examined 8 strains and found the flagellation to be lophotrichous with flagella arising from one pole or occasionally from both poles. Cottew (1950) using the Kirkpatrick stain examined 2 strains isolated from sheep and found many flagella arising from one pole. Lajudie, Fournier and Chambon (1953) examined several strains using the Caeser-Gil stain and found 1 to 4 flagella arising from one pole except in cells undergoing binary fission, when flagella could be seen arising from both poles. Wetmore and Gochenour (1956) examined 15 strains in the electron microscope and concluded that one or more flagella may arise from one or both poles. Egerton (1963) studied 1 strain and found a single flagellum at one pole. Laws (1964) using the Kirkpatrick stain examined 74 strains and found they had a single flagellum at one pole. Although the present investigation, using the electron microscope, examined a number of strains growing in the exponential phase, the bacterial cells were multitrichous and the flagella were always seen to arise

from one pole except in paired cells or cells about to divide, when flagella could be seen at the free ends of the cells. It is possible that the monotrichous polar flagellation observed by some investigators could have been due to breakages of the tuft of flagella during preparation of cultures for microscopic examination, because in the present work, free flagellar elements could be seen in even the most carefully handled specimens, and in any specimen there were always some cells having no flagella. The peritrichous flagellation reported by one worker (Sakihara, 1952) is puzzling. It is possible that mistakes could have been made by using the ordinary light microscope to study the implantation of flagella. Detached flagella which are numerous in specimens, lying on or around cells would appear to arise from such cells. Ps.aeruginosa has been found to possess a single flagellum at one pole and some workers (Gilardi, 1968; Sutter, 1968) have considered this to be an important criteria in differentiating it from Ps.pseudomallei as they have found the latter possessing more than one flagellum arising from one pole. The findings in this present study would also point towards its usefulness in the routine diagnostic laboratory.

The presence of surface appendages similar to fimbriae (pili) reported for some members of the Enterobacteriaceae (Brinton, Buzzell and Lauffer, 1954; Duguid, Smith, Dempster and Edmunds, 1955; Duguid and Gillies, 1957; Duguid and Wilkinson, 1961; Thornley and Horne, 1962; Duguid, Anderson and Campbell, 1966;

Duguid, 1966; Hoeniger, 1965 and Meynell and Lawn, 1967), *Vibrio spp.* (Tweedy, Park and Hodgkiss, 1968) and for *Pseudomonas* species (Houwink and van Iterson, 1950; Bradley, 1965 and 1966; Tweedy *et al.*, 1968 and Fuerst and Hayward, 1969) has not been reported for *Ps.pseudomallei*. Plate 1 shows appendages similar to the fimbriae observed in some species of the *Enterobacteriaceae* and *Pseudomonadaceae*. The reason for the low proportion of fimbriated cells in the strains examined is not clear, but Duguid *et al.* (1955) thought that the low proportions of fimbriated cells in cultures of *E.coli* might be associated with culture conditions that do not selectively favour the growth of fimbriated cells which may arise through spontaneous mutations. It is possible that the culture conditions in the present experiments did not enhance the production of fimbriate cells but promoted the growth of cells in the non-fimbriate phase.

Fimbrial agglutination has been observed in some members of the *Enterobacteriaceae* (Duguid *et al.*, 1955). In the present study a few randomly selected strains were tested using guinea-pig, fowl, sheep and horse blood corpuscles. The failure to demonstrate visible agglutination may not necessarily be due to the absence of a haemagglutinating property but may be a quantitative effect due to the low proportions of fimbriated cells in the suspensions used.

Duguid *et al.* (1955) and Duguid and Gillies (1957) also demonstrated an adhesive property of fimbriated cells to a wide range of cellular materials and theorised

on the possible role of fimbriae as organs of attachment that may permit the bacterium to attach to a possible site of entry to the susceptible host. If this has a foundation then it may also have a bearing on the mode of attachment and entry of Ps.pseudomallei - an organism considered by many workers to be a soil inhabitant like Ps.aeruginosa and whose common mode of entry into a susceptible host is thought to be through cuts, burns and abrasions.

(b) Cultural variation and colonial characters

The findings have shown that the colonial morphology of Ps.pseudomallei in cultures originating from old laboratory strains or from relatively recent isolations may vary and be associated with a multiplicity of colonial forms often resulting in the occurrence of more than one type of colony and sometimes as many as three distinct types in a single culture. Irrespective of the medium, a minimum period of 48 hours' incubation has been found necessary to allow for the full development and differentiation of many of these colonial forms. Altogether, six morphological types have been recognised which for convenience of description have been designated as 'S', 'SR', 'R', 'Rr', 'M' and "dwarf".

Their distribution on initial plate cultures suggested that certain colonial types ('S', 'SR' and 'R') are common and may possibly be the basic types and others such as the 'M' and the 'Rr' types may be uncommon variants. However, if serial subcultures of single

colonies derived from cultures showing either one single colonial type or many types are carried out, any of the types may give rise to a proportion of colonies of the other types. Identical colony types have not always yielded similar secondary dissociants. Variations have been seen in the range and yield of secondary dissociants and one 'SR' type primary colony has been found to yield a 'dwarf' rough yellow colony similar to those seen when some 'SR' type cultures are plated with low multiplicity doses of phages to which they are otherwise sensitive (See: Part 4). The secondary dissociants, although reproducible through successive serial subculture, were not always stable. They were found to yield dissociants which sometimes re-dissociated to the parental type. The 'R' and the 'Rr' types have been shown to be the most stable. Perhaps the most significant finding in this work is the behaviour of 'M' type strains which have been shown to be rapidly autolytic in broth or agar cultures causing high alkalinity in the medium as it became sterile.

The cause of these colonial dissociations is not clear. Some may, as suggested for some other bacteria, represent non-genetic variations and others may represent phenotypic expressions of genetically altered variants that have arisen through spontaneous mutations (Braun, 1947), lysogenic conversions (Coetzee & Hautrey, 1962; Jones, McDuff & Wilson, 1962; White, Foster & Lyon, 1962; Zierdt & Marsh, 1971) or due to "pseudolysogeny" (Martin, 1973). A high incidence of lysogeny in

Ps.pseudomallei has been found in the present study (See: Part 4).

From the medical microbiology point of view, these dissociations must cause some concern. In a number of publications originating from clinical laboratories the recognition of Ps.pseudomallei in primary cultures has in the main depended upon the colonial appearance which has been stated as "rough" and "corrugated". Only a few papers have mentioned other colony types that may occur in addition to "rough" types (Grant and Barwell, 1943; Gutner and Fisher, 1948; Beamer et al., 1948; Cox and Arbogast, 1945; Sakihara, 1952; Nigg et al., 1956; Ertug, 1961; Zierdt and Marsh, 1971; Rogul and Carr, 1972). Many of these descriptions have been so vague that it causes uncertainty as to whether the 'rough' corrugated colonies of different workers were a uniform colony type and whether the 'smooth' or 'mucoid' forms mentioned by other workers were genuine types or whether they have been confused for one or the other. There is also a possibility that some of these descriptions have been based on morphological appearances observed before colony differentiation had taken place. There has been some confusion in terminology; for example, Minett (1959) assumed that the 'corrugated' types of Stanton and Fletcher (1925a,b and 1927a) correspond to the colonies described as 'rough' by Nicholls (1930) and Finlayson (1944). There is an indirect assumption that the 'rough' colonial type of Nicholls (1930) corresponds to the 'rough' type of

Finlayson (1944) but this is incorrect. The 'rough' colonies of Nicholls (1930) would appear to correspond to the 'ultracorrugated' colonies of Stanton and Fletcher (1927) and to the 'R' colonies met in the present study since these were all found as wrinkled and corrugated colonies much earlier in the course of incubation than the 'SR' type. Moreover, they were found to be tenacious so that a colony could be picked up intact from the surface of the medium. On the other hand, the 'rough' type of Finlayson (1944) would appear to correspond to the 'corrugated' and rugose colonies of Stanton and Fletcher (1925a,b) and to the 'SR' type colonies met in the present study as there has been no mention by either worker of the tenacious character of the colonies. Both Stanton and Fletcher and Finlayson described the colonial appearance within 24 to 48 hours' incubation as round, opaque with dome-shaped centres and transparent, flattened, crenated margins, some colonies showing a surface umbilication.

Minett's (1959) interpretation that the 'mucoid' colonies of Stanton and Fletcher (1925a,b) correspond to the 'smooth' variant of Nicholls (1930) and Finlayson (1944) may be correct but one must not be led to the conclusion that distinct 'smooth' and 'mucoid' types do not occur. Indeed, Nicholls recognised variants from his 'smooth' cultures which he termed "suicidal" types because they tended to die out within 10 days. These correspond with the "M" type cultures observed in the present work which showed an autolytic

tendency and were of low viability. Although there has been no mention of autolytic tendencies, Sakihara (1952) and LeGac et al.(1954) and Zierdt and Marsh (1971) have also found "mucoid" strains in addition to "smooth" and "rough" types. Borchardt et al. (1966) found neither "smooth" nor "rough" colonies but only a "mucoid" type.

Thus it would seem that 4 of the 6 types of colonies found in the present study have been met by previous workers and it is likely that the remainder of the types found in the present study have also been met by previous workers. The 'Rr' type found as a dissociant among 'S' type colonies corresponds to the "parchment-like" intermediate colony that Nicholls (1930) derived from his 'rough' colonies since the parchment-like surface texture has been a feature of the 'Rr' type colonies as well. Similarly, the 'dwarf' rough yellow colonies encountered in the course of serial subculture of 'SR' colonies may have similarities with the virulent, small, 'rough' yellow lacy colonies that Nigg et al. (1956) derived in serial mouse passage of relatively less virulent large 'rough' colonies. Zierdt and Marsh (1971) considered that dissociation in A.mallei and Ps.pseudomallei parallels that reported for Ps.aeruginosa (Zierdt and Schmidt, 1964). Among 18 strains of Ps.pseudomallei they found 15 strains giving rise to more than one colony type. Two of these had each yielded 4 colony types and a further 2 strains had each yielded 5 colony types.

The occurrence of uncharacteristic colonial types

not having the typically corrugated appearance (and lacking in characteristic odour and metallic sheen) might suggest to the bacteriologist unfamiliar with Ps.pseudomallei that he is dealing with some completely unrelated organism which may be rejected as a non-pathogen or as a contaminant.

(c) Odour and metallic sheen

An earthy odour and a metallic sheen are features which have been recognised as characteristics of cultures of Ps.pseudomallei (Minett, 1959; Wilson and Miles, 1964). A variety of descriptions of the odour produced by cultures of Ps.pseudomallei on media such as nutrient agar, blood agar and trypticase soya agar, is scattered through literature and includes such terms as "earthy" (Cottew, 1950; Rimington, 1962; Laws, 1964), "sweetish" (Retnasabapathy, 1959), "mouldy" (Ketterer and Bamford, 1967; Gutner and Fisher, 1948), "musty" (Gilardi, 1968; Stokes and McCarthy, 1969; Kingston, 1971), "reminiscent of eucalyptus" (Grant and Barwell, 1943), "aromatic, piquant" (Zierdt and Marsh, 1971), "pungent odour devoid of ammonia" (Beamer et al. 1954), "earthy, ammoniacal" (Rowlands and Curtis, 1965; Greenawald et al., 1969) and "strong, musty, earthy odour" (Salisbury and Likos, 1970), "earthy" odour (Osteraas et al., 1971).

Many others who have reported on isolations of Ps.pseudomallei have made no mention of any distinct odour. Finlayson (1944) is the only worker to have noted the presence of an "aromatic" odour in the "rough"

variant and the absence of such in the "smooth" variant of a culture of Ps.pseudomallei (B.whitmori).

Souchard (1932) was perhaps the earliest to note the metallic lustre of cultures of Ps.pseudomallei (B.whitmori) on nutrient agar. Since then a sheen, described variously as "aluminium paint", "beaten-copper", "oily, iridescent", etc., have been found by several including Grant and Barwell (1943); Gutner and Fisher (1948); Cottew (1950); Wilson and Miles (1964); Green and Tuffnell (1968) and Kingston (1971). Beamer et al. (1954) also noted this character but found it to be variable and surprisingly enough the lustre has also been observed by them on cultures grown on salt agar containing 4%-6% NaCl, which are concentrations found in the present study to be inhibitory to all strains of Ps.pseudomallei though not inhibitory to Ps.aeruginosa.

The present work has shown that both these characters are common features of the 'SR' type cultures and that the characteristic odour (sweet, aromatic) may also be detected in 'R' and 'Rr' type cultures. Young cultures of the 'S' and the 'M' type were not found to exhibit either of these characters. Finlayson (1944) also found his "smooth" variant lacking in any aromatic odour. It is likely that those who encountered one or both of these characters were handling cultures that were neither "smooth" nor "mucoid".

(d) Chromogenic characters

The production of water soluble, diffusible pigments

by Whitmore's bacillus has been reported by a few workers. Gary and Koch (1951) mentioned a bluish-green pigment produced by their strain and Brygoo and Richard (1952) reported that some strains of Malleomyces pseudomallei produce a yellow water soluble pigment which may be extracted by acid treatment. A 'rough' corrugated strain studied by Colling, Nigg and Heckly (1958) produced a lavender pigment. The strain examined by Gary and Koch (1951) was subsequently shown by Haynes (1951) to be Ps.aeruginosa.

Several other workers (Grant and Barwell, 1943; Cottew, 1950; LeGac et al., 1954; Ziskind et al., 1954; Beamer et al., 1954; Wetmore and Gochenour, 1956; Nigg, Ruch, Scott and Noble, 1956; Ertug, 1961; Laws, 1964; Green and Tuffnell, 1968; Gilardi, 1968; Stokes and McCarthy, 1969; Zierdt and Marsh, 1971) have apparently looked for but have been unable to detect any diffusible pigments. A range of colony colourations (white, cream, yellow, bright orange, cafe-au-lait, brown, tan, rosy or red) have been noticed usually after prolonged incubation. The present results also indicate the absence of diffusible pigments on an ordinary culture media. Sixty-nine of the cultures grown on 2% sodium chloride agar developed bright orange colony colourations after 5 to 6 days' incubation. The 6 Ps.aeruginosa cultures became purplish in colour. It would point to the influence of salt constituents in the medium as a cause of some of the colour variations possibly through the interaction between such salts and

the bacterial metabolites.

(e) Biochemical characters and carbohydrate oxidation

The results, particularly in respect of "sugar" reactions, have not shown agreement with those of other workers whose findings have been summarised in Appendix C. The strains have shown an inertness in their activity towards the 'sugars' used. An oxidative activity was observed with glucose but some strains required longer periods of incubation to show reactions. The inactivity or the low activity towards the 'sugars' may be due to the age of the cultures (many of them being old laboratory cultures), but this may not be true for all cultures, particularly those from Sabah, Malaysia, which were comparatively recent isolates. Part of the failure therefore may be due to other factors. Wetmore and Gochenour (1956) considered that the peptone content in the basal medium for sugars could be rapidly utilised by the organism thereby causing a shift to alkalinity. They found a basal medium low in peptone content to be more satisfactory but concluded that reactions to 'sugars' and a number of other biochemical tests (i.e. MR; V-P; Citrate; Indole, etc.) largely designed for the identification of 'coliforms' to be of little value in the differentiation of strains within the species and in differentiating Ps.pseudomallei from Ps.aeruginosa. Even though Wilson and Miles (1964) have classified the two species under different genera (Loefflerella and Pseudomonas respectively), a scrutiny

of the biochemical and 'sugar' reaction tests will show the degree to which the two species share the "positive" and "negative" reactions listed under each. The findings in the present study would therefore add support to the observations of Wetmore and Gochenour (1956).

Tests recommended by Cowan and Steel (1965) for the preliminary identification of Pseudomonas in addition to tests to determine the ability or inability to grow on CTA agar, 4% NaCl agar, to oxidise potassium gluconate may be more useful in identifying Ps.pseudomallei than a gamut of tests laid down in many of the current textbooks. The results may undoubtedly take considerable time to yield conclusive results and it is perhaps here that antimicrobial sensitivity patterns, bacteriophage affinities (see: Part 4, p.234) and serology (see: Part 5, p.270) may aid in the rapid and more accurate identification of the organism.

(f) Haemolytic activity

Wetmore and Gochenour (1956) in a comparative study of the genus Malleomyces (which included 15 strains of M.pseudomallei) and various Pseudomonas species found the strains of M.pseudomallei to be non-haemolytic. They tested this character by plating on tryptose soya agar containing 10% sheep or rabbit blood. The period of incubation was not mentioned. Wilson and Miles (1964) stated that Loefflerella pseudomallei is non-haemolytic on horse blood agar incubated for 72 hours at 37°C. Minett (1959) stated that the organism

is feebly or not at all haemolytic but does not mention the culture conditions (medium and period of incubation). Liu (1957) in a survey of haemolysin production among Pseudomonas species found his strains of Ps.pseudomallei to be haemolytic on brain-heart infusion agar containing 5% suspension of human erythrocytes (washed cells restored to original concentration in blood) when such plates are incubated for 48 hours. However, the haemolysis was found only in areas where the colonies were crowded and not around isolated colonies growing in less crowded areas of the plates. Liu (1957) also found that strong haemolytic reactions were not obtained on similar medium using rabbit, guinea-pig or sheep erythrocytes. Green and Tuffnell (1968) describing the bacteriology of a strain freshly isolated from a human infection, found a "beta-type" haemolysis after 5 days' incubation on blood agar. They too found the haemolysis to be restricted to areas where the growth was heavy and absent around individual colonies growing in isolation. Cottew (1950), Rimington (1962) and Kingston (1971) using sheep blood agar observed a similar haemolysis in 3 and 5 days' incubation, respectively. The haemolysis was marked particularly in the area of the original inoculum. Lim and Retnasabapathy (1967) using ox blood agar observed a similar haemolysis after 24 hours' incubation at 37°C.

The present study was revealed that a considerable number of the strains of Ps.pseudomallei cause a discernible haemolysis after 72 hours' incubation at 37°C on tryptone

soya agar containing 5% horse blood. Sheep blood was found to be less satisfactory for the demonstration of this haemolytic activity. On the other hand, the haemolytic activity of the Ps.aeruginosa strains used in the study was greater than that of Ps.pseudomallei and was most marked when they were grown on a medium containing sheep blood.

The haemolysis caused by Ps.pseudomallei was, in many respects, similar to that reported by Liu (1957) and Green and Tuffnell (1968) since it occurred in areas where the growth was confluent or the colonies were not completely isolated thus making it difficult to recognise any particular colonial types that may be responsible for the haemolysis. However, on occasions, it was possible to associate the haemolysis in these areas with 'dwarf' rough yellow colonies which were rarely detectable as isolated colonies in such cultures. Such 'dwarf' colonies occurred frequently and were seen isolated from the other colonial types when certain strains of Ps.pseudomallei were mixed with low multiplicity doses of some phages to which they were sensitive and then inoculated on to blood agar plates.

Liu (1957) considered the possibility that the production of haemolysis in areas of dense bacterial growth on blood agar might be due to an alteration in the metabolic pattern through the mutual inhibition of the organisms growing in a dense population. The present study would suggest that phage activity may be involved in haemolytic activity.

The findings in the present study have indicated the possibility of the occurrence of a heat-stable and a heat-labile haemolysin. These were more easily demonstrable in the cell-washings of 6-day cultures grown on cellophane-layered agar medium than in the culture supernates of the liquid medium. Liu (1957) was able to demonstrate high concentrations of haemolysin in the washings from 1-3 day cultures grown on cellophane-layered tryptone agar containing 1% glucose. The differences may be due to the medium constituents and the erythrocyte species used in the titrations. Liu (1957) used 'Difco' tryptone compared with tryptone soya agar (Oxoid) containing 0.25% glucose used in the present study. Liu (1957) found glucose to enhance the production of haemolysin and moreover, he used a 10% suspension of human erythrocytes because of the greater sensitivity (as observed by Liu) of this species of erythrocytes to the haemolysin.

However, the present study has shown that a haemolytic activity comparable to that of the washings of 6-day cellophane grown cultures could also be found in aqueous extracts of disrupted cells prepared from 3-day cultures grown by the cellophane technique. As with the cell-washings, the heating of the aqueous extracts resulted in a partial loss of the haemolytic activity indicating the possible presence of a heat-stable and a heat labile haemolysin. Whether these are true intracellular haemolysins or whether they are those that make up 'extracellular' haemolysins, have not been determined

but, if they are, then their effects through autolysis or phage induced lysis of cells, could be considerable. The incidence of lysogeny in Ps.pseudomallei has been shown to be very high (see: Part 4) and a fraction of the cell population of any lysogenic strain is known to produce phage and lyse in the course of releasing these infectious particles.

(g) Toxicity of culture preparations

The production of an exotoxin from Ps.pseudomallei was first reported by Legroux, Kemal-Djemil and Jeremac (1932). Subsequently, Nigg, Heckly and Colling (1955) demonstrated the lethal toxicity of bacteria-free culture filtrates of 8-10 day cultures grown in beef extract broth containing glycerol. They also showed dermo-necrotic reactions by inoculating the filtrates intradermally into a guinea-pig. Colling, Nigg and Heckly (1958) described the cultural conditions that influenced the production of the exotoxin, the thermolabile character of the toxin and the differing toxigenicities of different strains although there was no correlation between toxigenicity and colonial morphology. Heckly and Nigg (1958) found that culture filtrates similar to those of Nigg et al. (1955) contained 2 thermolabile exotoxins, one lethal and the other dermo-necrotic, and a thermostable endotoxin. Heckly (1964) made further attempts to characterise the thermolabile exotoxins since they appeared to involve other biologically-active substances and Heckly and Klumpp (1964) reported

that the necrotoxicity was associated with the enzymic activity (proteolytic and anticoagulant) of the organism.

Liu (1957) in a study of haemolysin production was able to demonstrate a lethal toxicity to mice of unheated culture filtrates of cell washings of 1-3 day cultures grown on cellophane-overlaid tryptose glucose agar. Subsequently, Liu (1969), in a study of the extracellular antigens of pathogenic pseudomonads, demonstrated the cutaneous reactions in rabbits elicited when such unheated filtrates of Ps.pseudomallei prepared from 24 - 30 hour growths (on cellophane-overlaid agar containing casaminoacids and glycerol) were injected intradermally.

Because of restricted animal house facilities, the material used in the present investigation for the study of toxicity had to be restricted to heated (100°C for 15 minutes) culture preparations. The aliquots were those from preparations made for the study of haemolysins (see p. 79). The low mortality in mice and the absence of cutaneous reactions in the rabbit used, are therefore understandable since the materials are devoid of thermolabile toxins.

The occurrence of 50% mortality in the heated culture preparations from a 6-day cellophane grown strain (strain 33-SR) and a similar result found for an aqueous extract of a 3-day cellophane-agar grown culture suggests the occurrence of a heat-stable toxin. It is possible that this is an endotoxin since the cell-washings from 6-day cellophane-agar cultures cannot be considered to be free of intracellular materials. The centrifugation

procedures may not have sedimented all particulate matter. The reason for the apparent lack of toxicity of materials from other strains obtained by these two procedures is not clear. Different degrees of toxigenicity due to strain differences have been pointed out by Colling et al. (1958) but these pertain to exotoxins. It is therefore possible to consider that the differences in toxicity (often an absence of toxicity) of the strains are dependent on the amounts of cellular lysis that has taken place during growth or during the freezing and thawing procedures. A heat stable (121°C for 30 minutes) endotoxin in aqueous extracts of disrupted cells has been demonstrated by Rapaport, Millar and Ruch (1961).

(h) Sensitivity to antimicrobial agents

(i) Disc diffusion: Von Gravenitz and Redys (1968) presented data that suggested the usefulness of drug resistance patterns in identifying Gram-negative, non-fermentative bacteria of medical significance not commonly recognised in the average clinical laboratory. Brundage et al. (1968), describing the bacteriology of 4 strains of Malleomyces pseudomallei isolated from human melioidosis,mentioned that their sensitivity to some commonly used antibiotics was uniform and sufficiently characteristic to serve as a criterion in the identification of the species. Thin et al (1970) using "Multodisks" (Oxoid) found their 10 strains to be sensitive to chloramphenicol, tetracycline, novobiocin, ampicillin

sulphatriad and neomycin and resistant to pencillin, methicillin, cloxacillin, fucidin, kanamycin, carbenicillin, polymixin B and gentamycin. Gilardi (1971) demonstrated the usefulness of antimicrobial sensitivity patterns in differentiating Gram-negative, non-fermentative bacteria which included 200 strains of pyocyanogenic Ps.aeruginosa, 51 strains of apyocyanogenic Ps.aeruginosa, 5 strains of Ps.pseudomallei and 561 strains belonging to species of four other genera. He tested these strains against several antimicrobial agents in the form of discs ("Sensi-discs" BBL) using the method of Bauer, Kirby, Sherris and Turck (1966) and found that 100% of the pyocyanogenic strains of Ps.aeruginosa were sensitive to polymixin B and gentamycin, 87% to neomycin, 20% to streptomycin, 11% to chloramphenicol, 3% to kanamycin and 2% to tetraycline. All these strains were found to be resistant to penicillin, ampicillin, erythromycin and nitrofurantoin. The apyocyanogenic strains of Ps.aeruginosa had shown a slightly greater degree of sensitivity to some of these antibiotics and all were found to be sensitive to polymixin and gentamycin, 72% to neomycin, 58% to kanamycin, 32% to streptomycin, 22% to tetracycline, 12% to chloramphenicol, 8% to ampicillin and 4% to erythromycin. All these apyocyanogenic strains were resistant to penicillin and to nitrofurantoin. The sensitivity patterns of the 5 strains of Ps.pseudomallei were found to differ markedly from those observed for Ps.aeruginosa and a

number of the other Pseudomonas species. All the strains of Ps.pseudomallei were found to be sensitive to tetracycline, ampicillin and kanamycin and 80% were found sensitive to neomycin and to chloramphenicol. All strains were found to be resistant to polymixin, gentamycin, streptomycin, penicillin, erythromycin and to nitrofurantoin. The pattern of sensitivity of Ps.pseudomallei observed in the present study is similar to that observed by Gilardi (1971) although his method used different disc-potencies of antimicrobial agents in some cases. If one considers all inhibitory zones of diameters greater than 12 mm as indicative of sensitivity then 83.5% of the strains examined are sensitive to tetracycline (disc-potency 10 µg.), 100% to tetracycline (disc-potency 50 µg.), 91.8% to ampicillin, 78.6% to chloramphenicol, 13.1% to gentamycin, 3.3% to streptomycin, 3.3% to erythromycin and none to polymixin, penicillin and nitrofurantoin. The sensitivity pattern shown by the strains in the present work deviated from that seen by Gilardi (1971) in the sensitivity of some strains to gentamycin, erythromycin and streptomycin. Only 1 strain showed sensitivity to colistin methane sulphonate but this cannot be compared with the results of Gilardi (1971) since he did not test his strains against this agent. The 2 strains (or 3.3% strains) of Ps.pseudomallei in the present study that were found to be inhibited by streptomycin were strains 15 and 113, both of which were strongly inhibited with zones of 30 mm and 24 mm diameter, respectively. The same two strains

were also sensitive to erythromycin. It is also interesting that strains 15 and 113 were 2 of the 3 strains found to be strongly sensitive to gentamycin. The antibiotic sensitivity pattern of these two strains might therefore lead one to doubt the identity but the morphological, cultural, biochemical, serological and bacteriophage characteristics were all indicative of the strains being genuine Ps.pseudomallei. Streptomycin sensitive and chloramphenicol resistant strains were also reported by Kingston (1971) and Robinson and Ballion (1966). Retnasabapathy (1959) using Evans' "Sentests" found his 2 strains sensitive to streptomycin. The history of strain 15 shows that it was isolated in Ecuador and as no other cases of melioidosis have been reported in that country, it is possible that this culture is the one isolated by Biegeleisen et al. (1964) who showed that the organism was sensitive to 1.5 µg./ml of streptomycin. Our MIC studies against strain 15 have shown it to be one of 2 strains in the study sensitive to a low concentration of streptomycin. Ives and Thomson (1953) found their strain isolated in Central India to be sensitive to 50µg./ml of streptomycin.

The striking similarity in the present findings and those of Gilardi (1971) is the resistance of all strains of Ps.pseudomallei to polymixin. This agent is known to diffuse poorly in agar media yet quite large zones of inhibition were observed against the strains of Ps.aeruginosa used in the study. Equally striking is the resistance of strains of Ps.pseudomallei except

strain 15 to colistin methane sulphonate. In an earlier publication, Gilardi (1968) indicated the resistance of Ps.pseudomallei to colistin. A high degree of resistance of Ps.pseudomallei to colistin was also found by Farkas-Himsley (1968).

The findings on the sensitivity of the 4 strains of Ps.aeruginosa, however, show far less agreement with those of Gilardi (1971) particularly if zones of inhibition greater than 12 mm diameter are to be considered as sensitive. It would then indicate that 100% of the strains are sensitive to tetracycline (disc-potency of 10 µg.) and that 75% (3 strains) of the strains are sensitive to both streptomycin and chloramphenicol at disc-potencies of 10 µg. each. This may seem an unusual sensitivity pattern for Ps.aeruginosa even though strains sensitive to such antibiotics are known to occur. It is possible that the very limited number of strains in the present study is not representative of the typical and commonly occurring strains of Ps.aeruginosa.

Garrod and Waterworth (1971) and Castle and Elstub (1971) have indicated the lack of uniformity in the procedures used in Britain in antibiotic disc sensitivity tests. Various laboratories have differed in the use of a method, in its execution or in the interpretation of the results. Thus if disc-sensitivity pattern to antimicrobial agents is to be used as an aid in identifying Ps.pseudomallei as suggested by Gilardi (1971) and Poe et al. (1971), then more extensive investigations

with Ps.pseudomallei (and apyocyanogenic strains of Ps.aeruginosa) should be carried out by an agreed and uniform method that could, at the same time, be simple and economical for use in the developing nations where melioidosis is endemic.

In recent years, the Kirby-Bauer method (Bauer, Perry and Kirby, 1959; Bauer, Kirby, Sherris and Turck, 1966) has been increasingly used in the U.S.A. and a second method proposed by a working group of the World Health Organisation (Ericsson and Sherris, 1971) has gained recognition in Sweden, France, Germany and also in the U.S.A. Both methods have aimed at minimising the influence of a number of variable factors such as the composition of the medium, thickness of the medium, size of the petri-plates (15 cm diameter), growth phase and size of the inoculum, method of spreading the inoculum, size of the antibiotic disc and its potency, temperature and time of incubation and measurement of zones of inhibition. In the Kirby-Bauer method, the zones of inhibition are interpreted from tables that set out the limits of diameters for full and intermediate sensitivity and resistance for each antibiotic. In the method suggested by the working group of WHO, the zones of inhibition are measured and translated into minimum inhibitory concentrations (MIC) by reference to regression lines (plots of zone diameters against MIC for organisms of differing degrees of sensitivity) thus enabling the organism under study to be placed in a category of sensitivity according to

tables of 'break-points' (in terms of MIC) separating these categories. As pointed out by Garrod and Waterworth (1971), the preparation of regression lines is an enormous task and the practice of regulating the inoculum by an opacity standard cannot be expected from many hospital services. They also considered that the costs in using outsize plates and large volumes of culture media (as much as 80 ml/plate) can be prohibitive factors. The reluctance to accept such desirable reforms in disc-sensitivity testing is therefore understandable and a simpler yet uniform system seems wanting, considering also the needs of developing nations. Solutions to such a method may be found in the recommendations of Garrod and Waterworth (1971). They recommended that zone inhibitions be interpreted by comparison with a control culture of known normal sensitivity (i.e. the use of Ps.aeruginosa strain NCTC 10662 to compare the sensitivity of other strains of Ps.aeruginosa) such that a zone diameter equal to or larger than that of the control strain could be interpreted as 'sensitive' and a zone diameter smaller than that of the control strain by at least 4 mm could be interpreted as 'moderately sensitive' and a zone diameter <10 mm could be considered as 'resistant'.

The adoption of such a system for sensitivity testing of Ps.pseudomallei will require the use of an acceptable 'control' culture. The results may offer some guidance in the selection of such a strain although the method used did not minimise the variables rigidly,

i.e. cell populations in the 'standard' inoculum, etc., nor did it eliminate interference from possible inhibitors to sulphonamides and tetracyclines that might have been present in the medium. The adoption of the method of Garrod and Waterworth (1971) may serve two functions in the clinical laboratory - a diagnostic guide for the identification of Ps.pseudomallei and as an indicator of the drug of choice for therapy.

It must however be stressed that sensitivity to antibiotics could alter. As seen from the results, the sensitivity of individual strains could show wide variations to some antibiotics. These may be due to mutations. The indiscriminate and excessive use of antimicrobial agents may lead to the selection of mutants already present in the population or arising as a result of the use of such agents. Transmissible drug-resistance (R) factors observed in some members of the Enterobacteriaceae or the plasmid-determined resistance transduced by bacteriophages in Staphylococcus may also be found in Ps.pseudomallei. Although conjugation, a process that has appeared necessary for transmission of 'R factors' has not been demonstrated in Ps.pseudomallei, it is thought to occur in Ps.aeruginosa. Nor has the phage transduced resistance seen with staphylococci been demonstrated in Ps.pseudomallei, although the high incidence of lysogeny (and possibly multiple lysogeny) leads one to speculate whether or not such transduced drug resistance may occur in this species.

(ii) Minimum Inhibitory Concentration (MIC) of antimicrobial agents: Investigations on the MICs of antimicrobial agents against Ps.pseudomallei have been few and have been limited to the effects of a small number of agents and often confined to small numbers of strains derived mostly from cases of human melioidosis (Cros and Demarchi, 1950; Ives and Thomson, 1953; Brygoo, 1953b,c,d; Chambon, de Lajudie and Fournier, 1954; Moustardier et al., 1959; Borchardt et al., 1966; Brundage et al., 1968; Hobby et al., 1969; Grunberg et al., 1969; Franklin, 1969; Eichoff et al., 1970; Beaumont, 1970; Konopka, Lewis and Stieglitz, 1970; Alexander and Williams, 1971; Fisher et al., 1971; Zierdt and Marsh, 1971; Franklin, 1971 and Calabi, 1973). These studies have shown that strains of Ps.pseudomallei are generally sensitive to chloramphenicol, tetracycline, novobiocin, kanamycin and some of the sulphonamides. Many of the strains selected for the present study were those recently isolated from cases of melioidosis in domestic animals. In the main, the MICs of antibiotics against all strains in the study (including the NCTC strains) have corresponded with the mean values obtained by these previous investigators, with chloramphenicol and tetracycline (both at low concentrations) proving to be the most active. Moreover, the MIC of each antibiotic against the strains studied fell within a comparatively narrow range except for that observed with gentamycin.

A similar observation for gentamycin was reported

by Franklin (1971) in a study of 20 strains of Ps.pseudomallei. The MICs ranged from 5 µg./ml to 180 µg/ml. Waitz and Weinstein (1969) in a study of 9 strains found that MICs ranged from 17.5 µg./ml to 75 µg./ml and Eichoff et al. (1970) found little to no activity against the 10 strains studied by them. Thus, although the majority of the present strains have been found to be resistant or of low sensitivity to gentamycin, there may occur rare strains such as strain 15 (from Ecuador) and strain 113 (from Sabah) that may show a higher degree of sensitivity (5 µg./ml - 10 µg./ml) to this agent. A peak blood-level concentration of 5 µg./ml is achievable (Kirby and Standiford, 1969) and indeed a case of human melioidosis is claimed to have been successfully treated with this agent (Zimmerman, 1970). The occurrence of a minority of strains with a low degree of sensitivity to gentamycin and, even more rarely, the occurrence of a strain with a fairly high degree of sensitivity to this agent, cannot be dismissed without a comment. Franklin (1971) stated that the varying degrees of sensitivity to gentamycin, evident among the strains in his study, were not related to the time which had lapsed since the original isolation of the strains. The present findings indicate that except for strains 15, 32, 39 and 41, the only strains to show a degree of sensitivity to gentamycin were those which had been isolated more recently - the strains from Sabah.

Although carbenicillin and colistin at the

concentrations used were not inhibitory to any of the strains including strain 15, these results cannot be considered as meaningful. Trials with higher concentrations more related to those used in the disc diffusion studies need to be carried out to assess the susceptibility or resistance to these agents. As with results obtained for many of the other antimicrobial agents, the tube dilution tests against colistin and carbenicillin may perhaps then show some correlation to the findings in disc diffusion tests.

The results have helped to establish that strains of Ps.pseudomallei isolated from domestic animals are similar to those from other sources in respect of their sensitivity or resistance to antimicrobial agents. Aberrant strains sensitive to streptomycin, colistin and erythromycin may occur rarely and might cause confusion in identification schemes that include antimicrobial sensitivity patterns as aids. Nevertheless, antimicrobial sensitivity patterns may have their usefulness in differentiating a very large proportion of Ps.pseudomallei strains from Ps.aeruginosa.

Conclusions on the clinical efficacy of antimicrobial agents in the treatment of melioidosis cannot be drawn from the results since it is well known that agents which are more or less active in vitro may act differently in vivo. Besides, in vivo are those limited to a few therapeutic trials carried out on experimentally infected laboratory rodents (Miller, Pannell and Ingalls, 1948c; Cruickshank, 1949; Brygoo, 1953b,d; Chambon

et al., 1954; Hezebicks and Nigg, 1958; Khundanov, Devyatova, Padalko, Luk'yanova and Shkurko, 1961; Hobby et al., 1969; Fisher et al., 1969 and Grunberg et al., 1969) and these trials have not covered many among the wide range of perhaps more purer antimicrobial agents available today. Studies need also to be extended to test the efficacy of agents against naturally occurring melioidosis in domestic animals, that is, if we consider that the treatment of the disease in domestic animals (largely dependent on diagnosis) is important.

Part 3: Bacteriocin-like substances of Pseudomonas pseudomallei

1. INTRODUCTION

Since the finding of a pyocin (Jacob, 1954), a high proportion of Pseudomonas aeruginosa strains including those recovered from human infections have been shown to be pyocinogenic (Hamon, 1956; Holloway, 1960; Hamon, Veron and Peron, 1961; Papavassiliou, 1961; Wahba, 1963; Darrell and Wahba, 1964; Wahba, 1965; Paterson, 1965; Gillies and Govan, 1966; Govan and Gillies, 1969; Farmer and Herman, 1969). The increasing importance of Ps.aeruginosa as an "opportunist pathogen" particularly in hospital environments has led to the development of pyocin typing as an "epidemiological tool". Two such systems of typing have been devised. One (Osman, 1965) attempts to type strains on the basis of their sensitivity to a set of known pyocin producers and the other (Darrell and Wahba, 1964; Gillies and Govan, 1966; Govan and Gillies, 1969) attempts to type strains on the basis of their ability to produce pyocins to a set of known indicators. The latter system has also been extended to the typing of Ps.aeruginosa strains causing mastitis in cattle (Ziv, Mushin and Tagg, 1971).

Bacteriocinogenic strains have also been reported for some other Pseudomonas species (Hamon et al., 1961) but until recently, the occurrence of bacteriocinogenic strains of Ps.pseudomallei has not been investigated. This may have been mainly that the organism has only recently been recognised as a pseudomonad and partly to the recent revival of interest in melioidosis.

Published reports on attempts to detect antagonisms between strains of Ps.pseudomallei are limited to two recent papers, one from the U.S.S.R. (Tomov, 1970) and the other from the U.S.A. (Rogul and Carr, 1972). Tomov (1970) cross-tested 7 strains of Malleomyces pseudomallei by stab-inoculating the potential producer at the centre of a nutrient agar plate, allowing it to grow at 48-72 hours before killing it by exposure to chloroform and then overlayering the plate with an agar suspension of the strain to be tested for sensitivity. The test read after a further 24-48 hours' incubation, gave no inhibition of M.pseudomallei strains, but the same 7 strains were found to produce inhibitory zones of up to 35 mm diameter when M.mallei strains were used as indicators. One strain (selected because it showed the strongest inhibitory effect on M.mallei) was tested for similar activity on strains of Ps.aeruginosa, Escherichia coli, Salmonella typhi, Bact.dysenteriae, Pasteurella pestis, Past.pseudotuberculosis, Past. tularensis, Vibrio cholerae, Vibrio El-tor, Bacillus anthracis and 7 strains of soil bacilli and was found to inhibit the growth of all these strains.

Rogul and Carr (1972) surveyed 18 strains of Ps.pseudomallei (obtained from water, soil and animal sources in Vietnam, Thailand and Malaysia) for bacteriocin activity using the single streak method of Wahba (1963) on the medium of Darrell and Wahba (1964) modified to contain heart infusion broth (Difco) with 10^{-5}M iodoacetic acid, 0.1% sodium chloride, 0.1% K_2HPO_4, 0.001% phenol

red, 2% agar (Wahba agar). Eight strains were found to produce an agent which inhibited the growth of all other strains including themselves. They found that their "smooth" cultures as compared with the "rough" cultures from the same strain caused this inhibitory effect and they therefore considered the activity to be due to the toxic effects of ammonia generated by the "smooth" cultures. Although "rough" cultures were also found to produce ammonia, at the same time they found oxalic acid which may have neutralised the toxic effect of the ammonia. Rogul and Carr (1972) tested the same eight strains of Ps.pseudomallei for the antagonistic effect on Ps.mallei, Ps.cepacia and Ps.aeruginosa and found that Ps.mallei and Ps.cepacia were inhibited by "smooth" colony cultures and to lesser extent by "rough" cultures, but none of the Ps.aeruginosa strains were inhibited.

The antagonistic effects of Ps.pseudomallei on unrelated bacteria have been reported in two earlier publications. Jameson (1949) reported his findings of an "antibiotic from Pfeifferella whitmori". The activity spectrum was found to be similar to that of Penicillin. The preparation was found to inhibit Corynebacterium diphtheriae, Corynebact. hofmanni, Neisseria gonorrhoeae, N.meningitidis, N.pharyngis, Micrococcus tetragenus, Haemophilus influenzae, Staphylococcus pyogenes, Streptococcus pyogenes, Pneumococcus, Mycobacterium tuberculosis and Bacillus subtilis. Organisms found to be insensitive were

Shigella, Salmonella, Escherichia, Proteus, Pseudomonas and Streptococcus group D. However, some strains of group D streptococcus and some strains of "Bact. coli" of faecal origin were found to be sensitive to higher concentrations of the inhibitor. Jameson (1949) also tested the inhibitory effects of six NCTC strains of Pf.whitmori and found that 5 of these had activity spectra comparable with that of his original strain.

Fournier and Chambon (1958) have also briefly mentioned the antagonistic effects of B.whitmori cultures on Serratia, Aerobacter, Proteus, Escherichia, Streptococcus and Micrococcus species.

The present investigation, undertaken in 1970 was stimulated by the reports on pyocins produced by Ps.aeruginosa and by the claims on their usefulness in the typing of strains within the species. Bacteriocins, including pyocins, are defined as narrow spectrum antibiotics produced by strains within a species and acting usually on other strains of the same species. Thus, if bacteriocins with such degrees of specificity could be found in Ps.pseudomallei, it could not only provide a means for differentiating the strains within the species but also for differentiating the species from Ps.aeruginosa.

2. MATERIALS AND METHODS

(a) Strains of bacteria and their sources

(i) Ps.pseudomallei strains: All strains listed in Appendix A.1 were used in the initial screening tests for the detection of "producers" and their sensitive "indicators". Smaller batches from among them were used in subsequent experiments to detect the antibacterial activity to Ps.aeruginosa and other species of bacteria.

(ii) Ps.aeruginosa strains: The 5 strains listed in Appendix A.2 were used in cross tests against each other and against the strains of Ps.pseudomallei. The strains were also used in tests to determine their inhibitory activity on other bacterial species.

(iii) Other species of bacteria: Strains from 22 species of other bacteria were tested for sensitivity to antibacterial agents produced by Ps.pseudomallei and Ps.aeruginosa. The species were Escherichia coli, Alkaligenes faecalis, Enterobacter cloacae, Salmonella typhimurium, Salmonella typhi, Klebsiella aerogenes, Klebsiella pneumoniae, Shigella sonnei, Shigella dysenteriae, Pasteurella septica, Pasteurella haemolytica, Aeromonas hydrophila, Sarcina lutea, Haemophilus influenzae, Chromobacterium violaceum, Achromobacter anitratus, Serratia marcescens, Bacillus subtilis, Corynebacterium pyogenes, Staphylococcus aureus, Streptococcus faecalis and Streptococcus zooepidemicus.

(b) Media

"Oxoid" preparations of nutrient broth, nutrient agar, tryptone soya broth and tryptone soya agar were used in the study. Semi-solid nutrient agar (0.7% agar) was also prepared with "Oxoid" ingredients. Laked horse blood to a concentration of 20% was incorporated into culture media of test-plates when Corynebact. pyogenes or H.influenzae were to be grown.

(c) Ultraviolet irradiation of cultures

A Philips 15 watt germicidal lamp mounted within an inoculating cabinet[1] was used. For purposes of induction the cultures in uncovered petri-dishes were placed at a distance of 40 cm below the light source and exposed for 1-3 minutes. The dishes containing thin layers (2 mm approx.) of 18-hour broth cultures or their suspensions were rocked by hand during the exposure period. Subsequent manipulation of the irradiated material and their transfer to the incubator were carried out under subdued lighting.

For killing bacteria i.e. residual cells on primary streaks in the 'streak' test (p.170), the plates placed at a distance of 40 cm from the light source were exposed for 45 minutes.

(d) Mitomycin C as an inducing agent

Mitomycin C (Sigma) was added at the rate of 1.0 µg/ml of broth culture grown for 18 hours and were then

[1] Luckham Limited, Sussex, U.K.

incubated at 37°C for a further 6 hours.

(e) Thermostability of the inhibitory agents

1 ml samples of the material to be examined contained in 9 mm x 5½ cm glass tubes were heated in a waterbath at 60°C or 70°C for 30 minutes.

(f) pH of samples

pH values were determined using a pH meter[1].

(g) Electron microscopy

A simple negative-staining technique similar to that suggested by Bradley (1967) was used. A carbon-coated electron microscope specimen grid was inverted on the drop of the material to be examined and after 5-10 minutes, the grid was picked up and excess fluid removed by touching with a filter-paper. The grid was then allowed to dry for a few minutes and inverted on a drop of 2% phosphotungstic acid (pH 6.9) for 1 minute. After draining and drying, it was examined in the electron microscope[2] at magnifications ranging from 20,000 to 80,000.

(h) Screening procedures to detect antagonisms

Altogether, 3 different procedures were adopted:

(i) Method 1 ('spot-on-lawn' technique): Except for slight modifications, the procedure was similar to that used by Holloway (1960) for the detection of

[1] Pye Model 292 pH meter, Pye Unicam, Cambridge.
[2] Model AEI-EM 6B, Associated Electrical Industries Ltd., U.K.

pyocinogenicity and lysogenicity in Ps.aeruginosa. The strain to be tested for sensitivity was grown overnight in nutrient broth at 37°C and suspended at 1 in 100 in semi-solid nutrient agar which had been melted and cooled to 45°C. A 3 ml volume of this suspension was poured over the surface of a standard nutrient agar plate (9 cm diameter petri-plate containing 15 ml nutrient agar) and was allowed to set. The plate was then dried at 37°C for ½ hour before spotting with the strains to be tested for production of inhibitory agent. The test strains were grown overnight in nutrient broth at 37°C and a loopful of each culture was placed on the 'indicator' plate. Each 'indicator' plate provided sufficient space to test 16 strains and therefore several plates of the same indicator had to be used. The inocula of the strains to be tested were allowed to dry on the plates before incubating at 37°C for 24-48 hours. The plates were then examined for zones of inhibition around the spots.

(ii) Method 2 ('lawn-on-spot' technique): The strains to be tested for production were grown overnight in nutrient broth at 37°C. A loopful of each of 5 or 6 cultures was inoculated on to the surface of a nutrient agar plate. The inocula were allowed to dry before incubating at 37°C for 24-48 hours after which the bacterial growth was killed by exposure to chloroform vapour for 15 minutes. The plates were then re-exposed to air for 1 hour before over-layering each spot with approximately 0.1 ml of semi-solid nutrient agar suspension

of the indicator strain to be tested for sensitivity. The preparation of the agar suspension was the same as that used in the 'spot-on-lawn' method. The set of plates over-layered with the 'indicator' were then incubated at 37°C for 18 hours before examining for zones of inhibition.

(iii) Method 3: Except for some modifications, the method was similar to that used by Paterson (1965) for the detection and differentiation of bacteriocinogeny and lysogeny in Ps.aeruginosa. The strain to be tested for production was inoculated into nutrient broth and incubated at 37°C. Some cultures were induced (ultra-violet irradiation or mitomycin treatment) at 18 hours. The cultures grown for 24 hours (includes all induced cultures) and those grown for 48 or 72 hours were then centrifuged at 4000 r.p.m. for ½ hour. The crude supernatant fluid from each culture was recovered separately and was sterilised by adding chloroform (5% v/v). The chloroform suspended material was left either for 3-4 hours on the bench or overnight in the refrigerator before removing the chloroform. A standard drop (0.03 ml) from each of these sterile supernates was placed on a nutrient agar plate over-layered with semi-solid nutrient agar suspension of the strain used as the "indicator". Each plate provided sufficient space for placing 8 to 10 standard drops. After drying, the plates were incubated at 37°C for 18 to 24 hours and were then examined for confluent zones of inhibition

or for phage plaques over the area of the "drops".

(i) Plate assay to differentiate bacteriocin-like activity from phage activity

The sterile culture supernates found to cause zones of clear or opaque inhibition in tests by Method 3 were serially diluted in nutrient broth to give doubling dilutions ranging from 1 in 5 - 1 in 80 and ten-fold dilutions up to 10^{-6}. A standard drop (0.03 ml) of each dilution was placed on an 'indicator-plate'. The drops were left to dry before incubating the plates at 37°C for 18-24 hours. The plates were then examined for zones of confluent inhibition over areas spotted with the low dilutions and, for zones diminishing in intensity without plaque formation in the areas spotted with the higher dilutions.

(j) 'Streak' method to demonstrate bacteriocin-like effects

Strains found to produce bacteriophage/bacteriocin-like reactions in the screening tests were used in this study. The strains tested for sensitivity included those which had been found to be suitable as indicators as well as others picked at random. The method used was basically similar to those described for the detection of bacteriocins of Shigella (Abbot and Shannon, 1958) and of Ps.aeruginosa (Wahba, 1963; Gillies and Govan, 1966). The producer strain grown overnight at 37°C in nutrient broth or in tryptone soya broth

was inoculated as a streak (approximately 1 cm x 8 cm) across the centre of an 8 mm thick nutrient agar or tryptone soya agar plate. Sets of plates inoculated in this manner were incubated for intervals ranging from 24-72 hours. At the end of the incubation period, the growth on each plate was scraped off with a microscope slide (flamed and cooled prior to use). The plates were then exposed to chloroform vapour for 15 minutes to kill the residual cells and then re-exposed to air for 1 hour to evaporate any traces of chloroform. The strains to be tested for sensitivity were grown overnight in nutrient broth (or tryptone soya broth) at 37°C and diluted 1 in 100 in broth. A standard loopful (3 mm diameter) of the diluted broth culture was streaked halfway across the plate at right angles to the original inoculum. In this way it was possible to accommodate 7 to 9 strains on a plate by inoculating 4 or 5 strains on one side and 3 to 4 strains on the other. Large square petri-plates (10 cm x 10 cm) provided sufficient space to test 11 to 12 'indicators'. The plates inoculated with the 'indicators' were incubated at 37°C for 18 hours before reading results. The inhibitions were scored as follows:-

4 = Inhibition throughout length of secondary streak.

3 = No growth on secondary streak except at the end furthermost from the primary streak.

2 = Inhibition limited to an area of approximately 1 cm from the edge of the primary streak.

1 = Inhibition only on the area lying on the primary streak

(-) = No inhibition.

e = Partial inhibition amounting to a thinned growth.

r = Inhibition occurring but for a dense growth on and in the vicinity of the primary streak.

Thus "4e" would indicate a partial inhibition around the entire length of the secondary streak and "4er" would indicate a partial inhibition along the entire streak with an area of dense growth on and in the immediate vicinity of the primary streak.

The following modifications of this technique were tried in some experiments:-

(i) After scraping the bacterial growth from the primary streaks, the residual bacteria were killed by ultraviolet irradiation for 45 minutes instead of chloroform vapour. The method was similar to that used by Loverekovich, Loverekovich and Jenkins (1972) for the typing of Ps.aeruginosa by pyocin production.

(ii) Kohn's (1966) modification to the Wahba (1963) technique of pyocin typing in which the producer strain was grown on a sterile 2 cm x 7½ cm cellulose acetate strip ("Oxoid" Electrophoresis grade) placed over the surface of the growth medium in the petri-plate (8 cm diameter). After incubation, the strip with the bacterial growth on it was lifted off and discarded. The surface of the test-plate medium was then exposed to chloroform vapour for 15 minutes or to ultraviolet light for 45 minutes to kill any residual bacteria.

(iii) A procedure similar to above, but substituting

cellophane strips ("Viskin") for CAE membranes was tried.

(iv) The application of cultures of strains to be tested for sensitivity was made by means of filter paper strips instead of a wire loop. This helped to spread each 'standard' inoculum evenly. A sterile filter paper strip (3 cm x $\frac{1}{3}$ cm approx.) was charged by soaking in a broth suspension of the strain to be tested for sensitivity and, after draining the excess fluid, it was laid on the agar surface at right angles to the primary streak (producer strain) and immediately removed and discarded. A fresh strip was used for each inoculation.

(k) Antagonistic effects of Ps.pseudomallei strains on Ps.aeruginosa and on unrelated bacteria

The tests were similar to those used in testing the antagonistic effects of strains of Ps.pseudomallei on strains of their own species. A total of 70 strains of Ps.pseudomallei, picked at random, were screened for activity on Ps.aeruginosa (NCTC 5781) and on unrelated bacteria listed in Appendix A.3, using Methods 2 and 3 since Method 1 was found unsatisfactory. The simultaneous over-layering and "spotting" allowed many of the unrelated bacteria and the Ps.aeruginosa strains to outgrow the Ps.pseudomallei strains.

A total of 39 strains of Ps.pseudomallei including more than one dissociant from some strains were then used in the "streak" test to test their activity against

5 strains of Ps.aeruginosa listed in Appendix A2 and on unrelated bacteria.

(1) Antagonistic effects of Ps.aeruginosa strains towards each other and towards other bacteria including Ps.pseudomallei

One strain of Ps.aeruginosa was examined by Methods 1 and 3 for antagonistic effects towards 66 strains of Ps.pseudomallei and some strains of unrelated bacteria.

Five strains of Ps.aeruginosa listed in Appendix A2 were then tested by the "streak" method for antagonisms towards each other, towards 20 selected strains of Ps.pseudomallei (those used as "indicators" against other Ps.pseudomallei strains in the "streak" test) and towards other bacteria.

3. RESULTS

(a) Screening tests

(i) Method 1 ('spot-on-lawn' test): The reactions ranged from narrow zones (approximately 0.5 mm - 1.0 mm width) of clear confluent inhibition to much wider zones (approximately 2 mm - 4 mm width) of partial inhibition (Plates 25a & 25b). Of 116 primary strains tested, 92 were found to cause zones of inhibition on some of the strains against which they were cross-tested. The patterns of the strains inhibited by the producer strains were not the same in every case. One strain (strain 27) showed an inhibitory effect towards itself. Among the producer strains were found 3 that caused only the wide-zone type of partial inhibition. All other strains, with the exception of 10, caused narrow zone type of clear, confluent inhibition. The 10 strains caused both types of inhibition depending on the indicators used. These reactions are illustrated on Plates 25a & 25b where the strain 100 can be seen to have caused no effect on indicator strain 15 but has produced a narrow zone of clear, confluent inhibition on indicator strain 27 and a wide zone of partial inhibition on indicator strain 81. Strain 90 on the other hand has shown no effect on strains 15 and 27 but has caused a wide zone of partial inhibition on strain 81. Test-plates examined at 72 hours showed no changes in the size and intensity of the zones of inhibition except in a few instances where narrow-zone inhibitions were found to

develop further to reach a width of 4 mm - 8 mm (Plate 26). The transfer of chloroform-sterilised material from wide zones of inhibition into fresh indicator-plates, failed to cause inhibitory effects or to give rise to phage plaques but similar transfers from narrow zones of inhibition usually resulted in the formation of discrete plaques.

Many of the strains tested for sensitivity were not found to react or were found to react so weakly as to show hardly discernible zones of inhibition. However, 10 strains were found that showed a comparatively high degree of sensitivity. The results obtained against 10 such indicator strains are given in Fig.4 along with the results obtained by Method 3. These results show that indicator strains exhibited some variations in their sensitivity spectra. The resulting patterns, despite their overlaps suggested a heterogeneity of the strains in the collection. Broad groupings among the producer strains were evident (Fig. 5).

The test did not prove to be of much value in detecting the antagonistic effects of Ps.pseudomallei strains on other species of bacteria. The layering technique did little to prevent or minimise the growth of unrelated strains on the surface layer of the agar with the result that many of the unrelated species such as B.subtilis, Staph.aureus, Aeromonas hydrophila and E.coli tended to overgrow the "spots" of the Ps.pseudomallei strains rapidly.

(ii) Method 2 ('lawn-on-spot' test). The test was

Figure 4. A comparison of results obtained with Methods 1 and 3 using 116 strains of *Ps. pseudomallei*

narrow zone of inhition (Method 1)

wide zone of inhibition (" ")

phage plaques (Method 3)

nt not tested

negative

Producer strain	Indicator strain 2	4	13	15	17	27	33	74	79	83
1 1-SR										
2 SR										
3 SR										
4 S										
5 SR										
6 SR										
7 S										
8 M										
9 S										
10 S										
11 SR										
12 M										
13 S										
14 S										
15 M										
16 SR										
17 S										
18 M										
19 R										
20 S										
21 SR										
22 S										
23 S										
24 R										
25 SR										
26 SR										
27 SR										
28 S										
29 S										
30 SR										
31 SR										
32 R										
33 S										
34 SR										
35 SR										
36 S										
37 S										
38 S										
39 M										
40 S										
41 S										
42 1										
43	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
44 S										
45 SR										
46 S										
47 S										
48 M										
47 S										
48 M										
49 M										
50 SR										
51 R										
52										
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70 S										
71 M										
72 M										
73 S										
74 S										
75 M										
76 S										
77 SR										
78 M										
79 M										
80 M										
81 S										
82 S	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
83 S										
84 SR										
85 R										
86 SR										
87 SR										
88 SR										
89 SR										
90 SR										
91 S										
92 S										

40 S										
41 S										
42 M										
43	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
44 S										
45 SR										
46 S										
47 S										
48 M										
47 S										
48 M										
49 M										
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72 M										
73 S										
74 S										
75 M										
76 S										
77 SR										
78 M										
79 M										
80 M										
81 S										
82 S	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
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85 R										
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108 SR										
109 SR										
110 SR										
111 SR										
112 SR										
113 SR										
114 SR										
115 S										
116 SR										
117 SR										
118 SR										

Figure 5. A classification of 81 strains of *Ps. pseudomallei* on the basis of inhibition patterns with Method I against 4 'indicator' strains of *Ps. pseudomallei*

INDICATORS 2	4	15	27	PRODUCER STRAINS	GROUP
				28 34 44 49 58 69	A
				26 37 38 41 57 63 72 76 77 85 95 98 103 110	
				64 65 84	
				35 36 56 73 80 87 99 101 104 109 114 115 116	
				23 45	
				15 42 59 70 96	
				67 108	
				25 27 68 78 97 100	
				3 19 32 48 60 62 75 112	B
				9 16 47 50	
				46 111 113	
				4 81 83 102 106	
				14 18 21 22	C
				39 74	
				1 66 89 118	D

less satisfactory in detecting the antagonisms between strains of Ps.pseudomallei. Forty-eight strains were found causing reactions on other strains of Ps.pseudomallei and of these, only 8 caused zones of inhibition (wide,opaque or narrow and clear) free of scattered phage plaques. The others produced phage plaques alone or zones of inhibition with scattered phage plaques. The effects caused by 5 strains on 2 indicator strains are shown on Plates 27 and 28. Since all 48 strains were among those that had caused zones of inhibition in the tests carried out by Method 1, it suggested that the zones seen in the latter tests could have been due in part to the lytic activity of phages released by lysogenic strains.

Method 2, unlike Method 1, was of considerable value in screening for antagonistic effects of Ps.pseudomallei on the unrelated bacteria. Of 66 strains of Ps.pseudomallei tested, (20 of which were known not to exert any inhibitory activity on other Ps.pseudomallei strains), 58 caused varying degrees of inhibition on some of the unrelated strains (Aeromonas hydrophila, Past. septica, Staph. aureus and Sarcina lutea) used in this experiment. The varying degrees of inhibition caused by 11 strains of Ps.pseudomallei on Sarcina lutea are shown on Plate 29(11).

(iii) Method 3 ('drop-on-lawn'). The undiluted supernates from several induced and non-induced cultures caused zones of confluent or semi-confluent inhibition

on lawns of other Ps.pseudomallei strains (Plate 30) but confluent inhibitions could not be attributed to bacteriocin-like substances since the tests repeated with serially diluted culture supernates did not show zones diminishing in size and intensity with the increasing dilutions but instead, semi-confluent lysis or discrete phage plaques occurred at dilutions as low as 1 in 10. When tested against the 10 "indicator" strains used in Method 1 (see Fig.4), 76 (65%) strains were found to be lysogenic and with the exception of 4 strains, all of these fell into the group of 92 strains found producing confluent inhibition zones in the first method (Method 1). Often, the range of indicators attacked by a strain in tests with these two different methods was similar and occasionally, it was identical.

Supernates of cultures incubated for more prolonged periods (48-72 hours) also showed no bacteriocin-like activity but only a phage activity.

The results of the tests against the unrelated bacteria were different from those obtained against strains within the species Ps.pseudomallei. The supernates of 24-hour nutrient broth cultures of some of the strains of Ps.pseudomallei were found to exert a weak inhibitory activity on a few of the unrelated species. Of these, Past.septica and Sarcina lutea gave the most discernible sensitivity reactions. The effects of induced cultures were found to be no different from those of the non-induced cultures. More pronounced

inhibitory effects were observed in tests using the supernates of 72-hour broth cultures. Of the 70 strains tested, 58 strains caused zones of inhibition on Past.septica. The size and intensity of the zones of inhibition varied considerably suggesting that some strains of Ps.pseudomallei were stronger producers of the inhibitory agent than others. Those causing strong inhibitions on Past.septica showed a similar or slightly reduced inhibitory effect on Sarcina lutea, Aeromonas hydrophila, Past.haemolytica, Staph.aureus, Strep.zooepidemicus, Corynebact.pyogenes and B.subtilis. The effects of 7 strains of Ps.pseudomallei on some of these unrelated bacteria are shown on Plates 31, 32 and 33. By this method, the inhibitory activity of Ps.pseudomallei strains towards the other unrelated species of bacteria tested was found to be negligible or was absent. Attempts to concentrate the inhibitory agent by ultra-centrifugation or by suction filtration through cellophane sacs ("Viskin") failed.

Tests with serially diluted culture supernates on unrelated bacteria showed zones of inhibition which diminished in size and intensity as the dilutions increased. Phage activity was not seen. Maximum dilutions causing visible zones of inhibition were not found to exceed 1:100 even with the supernates from cultures of the apparently stronger producers. Plate assays of the activity of one such culture (Ps.pseudomallei strain 42-S) on several unrelated bacteria (Past.septica, Staph.aureus, B.subtilis, E.coli) are shown on

Plates 34, 35, 36 and 37. The inhibitory effects particularly those occurring at low dilutions were suggestive of the activity of a diffusing agent since the inhibition zones extended beyond the area of the respective "drops".

(b) Ps.pseudomallei strains tested by the "streak" method

The antagonistic effects of 48-hour non-induced cultures of 39 strains of Ps.pseudomallei on strains within the species and on strains belonging to other species of bacteria including Ps.aeruginosa are included in Table 14.

The strains of Ps.pseudomallei showed a far wider inhibition spectrum towards the unrelated bacteria. Although the zone inhibitions were not always uniform, all strains inhibited Past.septica, Past.haemolytica, Aeromonas hydrophila, Chromobact.violaceum, Haemophilus influenzae, Alkaligenes faecalis, Staph.aureus, Sarcina lutea. The activity towards B.subtilis, Streptococcus faecalis, Strep.zooepidemicus and Corynebacterium pyogenes were often more moderate than strong and occasionally they resisted the inhibitory effects of a few strains of Ps.pseudomallei, though not identical ones. Escherichia coli, Salmonella spp., Shigella spp., Klebsiella spp. and Enterobacter cloacae were often inhibited weakly and some strains of Ps.pseudomallei failed to exert any inhibitory effects on them. This appeared to be related to the occurrence of "weak" or "strong" producers among the

TABLE 14. The inhibitory effects of 39 strains of Ps. pseudomallei and 5 strains of Ps. aeruginosa when tested by the "streak" method against strains within the two species and against unrelated bacteria

PRODUCERS	INDICATORS	*B. subtilis*	*Staph. aureus*	*Sarcina lutea*	*Strept. zooepidemicus*	*Strept. faecalis*	*Corynebact. pyogenes*	*Past. haemolytica*	*Past. septica*	*Haemophilus influenzae*	*Alkalegen. faecalis*	*E. coli*	*S. typhimurium*	*S. typhi*	*Shig. sonnei*	*Shig. dysenteriae*	*Kl. aerogenes*	*Kl. pneumoniae*	*Serratia marcesens*	*Enterobact. cloacae*	*Achromobact. anitratus*	*Chromobact. violaceum*	*Aeromonas hydrophila*	*Ps. aeruginosa (NCTC)*	*Ps. aerug. (F.10)*	*Ps. aerug. (IC)*	*Ps. aerug. (HCr.5)*	*Ps. aerug. (HCr.13)*	*Ps. pseudomallei (2-S)*	*Ps. pseudomal. (2-M)*	" (4-S)	" (4-M)	" (9-S)	" (13-S)	" (14-S)	" (15-M)	" (17-S)	" (19-R)	" (20-S)	" (22-S)	" (24-R)	" (27-SR)	" (33-SR)	" (37-S)	" (67-S)	" (74-S)	" (79-M)	" (81-S)	" (102-S)	" (108-SR)
Ps. pseudomallei	2-S	2	3	3	2	1	2	3	3	1	2	1	1	–	1	1	1	–	–	–	1	2	2	2	1	1	1	1	–	–	–	1	1e	–	–	–	–	1e	1e	1e	–	1e	–	–	1e	–	–	–	–
"	2-M	2	4	3	2	1	2	3	4	1	2	1	1	1	1	1	2	1	·	1	–	2	4	2	1	1	1	1	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
"	4-S	1	2	3	1	1	1	3	3	1	2	1	1	–	2	1	1	–	–	–	1	2	2	–	–	–	–	–	–	–	–	–	1e	–	–	3e	–	–	1e	1e	–	–	–	–	1e	–	1
"	4-SR	2	2	3	1	1	1	3	3	1	2	1	1	–	2	1	1	–	–	–	1	2	2	–	–	–	–	–	–	–	–	–	1e	–	–	–	–	–	1e	1e	–	–	–	–	1e	–	1
"	4-M	2	2	3	1	1	1	3	3	1	2	1	1	–	2	2	1	1	–	1	1	2	2	–	–	–	–	–	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
"	8-M	2	3	3	1	–	2	3	3	1	2	1	1	1	1	1	1	–	–	1	1	2	2	–	–	–	–	–	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
"	13-S	2	2	3	2	2	2	3	3	1	2	1	–	–	1	1	1	–	–	–	–	2	2	–	–	–	–	–	–	–	3e	3e	–	–	–	–	1e	1e	1e	1e	–	3e	–	–	–	–	1e	1e
"	14-S	2	3	3	2	1	2	3	3	2	3	2	2	2	2	2	2	2	–	2	1	2	3	–	–	–	–	–	1	1	1e	1e	–	–	–	–	1e	1e	–	1e	–	–	–	–	1e	–	1e	1e
"	15-M	1	3	3	1	–	1	3	3	1	2	2	2	1	1	1	1	–	–	1	1	2	3	–	–	–	–	–	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
"	18-SR	2	3	3	2	–	2	3	3	2	2	2	1	–	1	1	1	–	–	1	–	2	2	–	–	–	–	–	1e	1e	4	4	–	–	–	–	4e	4e	4e	4e	4e	4e	4e	–	–	–	–	–	–
"	18-M	1	3	3	1	1	1	3	3	1	1	1	–	–	1	1	1	–	–	–	–	2	2	–	–	–	–	–	1	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
"	19-R	1	3	3	1	–	1	2	3	1	–	2	–	1	–	–	1	–	–	1	–	2	2	–	–	–	–	–	1e	1e	–	–	1e	–	–	1e	–	–	2e	2e	2e	–	–	–	–	–	–	1e	–
"	23-R	2	2	3	2	1	1	3	3	2	1	2	1	1	2	2	1	–	–	1	1	2	2	–	–	–	–	–	–	–	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1e	–
"	23-S	2	2	3	2	–	1	3	3	1	1	1	1	1	1	1	1	–	–	–	1	2	2	–	–	–	–	–	–	–	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1e	–
"	27-SR	2	2	2	1	1	–	2	2	1	2	2	–	–	–	1	1	–	–	–	–	1	1	–	–	–	–	–	1e	1e	1	1	–	1e	–	–	1e	–	1e	1e	1e	1e	1	2e	–	–	–	–	–
"	28-S	2	3	3	2	1	1	3	3	2	3	1	–	–	1	1	1	–	–	–	1	2	2	–	–	–	–	–	–	–	2e	2e	1e	1e	–	1e	–	2e	–	2e	2e	1e	1e	–	–	–	–	–	–
"	29-S	2	3	3	2	–	1	3	3	1	2	1	1	1	1	1	1	–	–	–	1	2	2	–	–	–	–	–	2	1	2e	2e	–	–	–	1	–	–	1e	–	–	2e	–	–	–	–	–	–	–
"	37-S	2	3	3	1	3	2	3	3	2	2	2	1	1	2	1	1	–	–	–	1	2	3	2	1	1	1	1	1e	1e	3e	2e	–	2e	–	2e	–	2e	–	2	2e	2e	–	2e	2e	2e	–	–
"	42-M	2	3	4	2	2	2	4	4	2	2	2	1	1	2	1	–	–	–	4	2	2	3	2	1	1	1	1	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
"	47-S	2	3	3	1	2	2	3	3	1	2	2	1	1	2	2	2	–	–	2	nt	2	3	–	–	–	–	–	1e	1e	2	2	–	4e	–	1e	2e	4e	–	2	2	1e	1	–	–	–	–	2e	–
"	50-S	3	3	3	1	1	2	3	3	1	2	2	1	1	2	2	2	2	–	2	nt	2	3	–	–	–	–	–	2e	2e	2e	2e	–	–	–	1e	2e	–	2e	2e	2e	–	2e	–	–	–	–	–	–
"	54-S	2	3	3	2	1	1	3	3	1	2	2	1	–	2	1	1	–	–	–	nt	2	3	–	–	–	–	–	–	–	1e	1e	–	–	–	–	–	2e	2e	2e	2e	2e	1e	–	–	–	–	–	–
"	57-S	2	2	3	2	1	2	3	3	1	1	1	1	–	1	1	1	–	–	–	nt	2	2	2	1	1	1	1	–	–	–	–	–	–	–	1e	2e	–	–	–	–	1e	1e	–	–	–	–	–	–
"	65-M	1	2	3	2	1	1	3	3	1	1	1	1	1	1	1	1	–	–	–	nt	2	1	–	–	–	–	–	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
"	66-M	1	2	2	2	1	1	3	3	1	2	1	1	1	1	1	1	g 1	–	1	nt	1	1	–	–	–	–	–	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
"	72-S	2	2	3	2	1	2	3	3	1	3	2	1	1	1	1	1	–	–	–	nt	2	2	–	–	–	–	–	–	–	1e	1e	–	–	–	–	–	1e	–	–	1e	–	–	–	–	–	–	–	–
"	75-M	2	2	3	2	1	1	3	3	1	3	2	1	1	2	1	1	–	–	2	nt	2	2	–	–	–	–	–	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
"	79-S	2	2	2	2	2	1	3	3	1	2	1	–	–	1	1	1	–	–	–	nt	2	3	–	–	–	–	–	–	–	1e	1e	1e	–	–	–	–	1e	–	–	–	3	–	–	–	–	–	–	–	–
"	79-M	1	2	2	1	2	2	2	2	1	2	1	–	–	1	1	1	–	–	–	nt	2	3	–	–	–	–	–	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	3	4	4	3
"	86-S	2	3	3	2	1	2	3	3	1	2	2	2	2	2	2	2	1	–	2	nt	2	3	1	1	1	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
"	92-S	2	2	2	1	2	1	1	3	1	2	–	1	1	2	2	1	–	–	2	nt	2	2	–	–	–	–	–	–	–	3e	3e	–	–	–	3e	–	–	–	–	4e	3e	–	–	–	2e	–	–
"	106-S	2	3	3	1	–	1	2	3	1	3	1	1	–	1	1	1	–	–	–	nt	2	2	–	–	–	–	–	–	–	–	–	–	–	–	–	1e	–	–	–	–	–	–	–	–	–	–	–	–
"	106-M	2	2	3	2	2	1	3	3	1	2	1	–	–	1	1	1	–	–	1	2	2	2	–	–	–	–	–	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
"	108-SR	2	3	3	2	2	2	3	4	1	2	2	2	1	2	2	2	1	–	2	1	2	2	–	–	–	–	–	1	1	–	–	–	–	–	–	1	–	–	–	1e	–	–	–	–	–	–	–	–
"	109-S	2	2	3	2	2	2	3	3	1	2	2	2	2	2	2	2	1	–	2	nt	2	2	–	–	–	–	–	–	–	1e	1e	–	1e	–	1e	–	1e	1e	1e	1e	–	1	–	1	–	–	–	–
"	110-SR	2	2	2	2	2	2	3	3	1	2	1	1	1	2	1	1	–	–	–	nt		1	–	–	–	–	–	2e	2e	–	–	–	1e	1	1	–	–	·	2e	4e	–	–	–	2e	2e	–	–
"	115-S	2	2	2	2	2	2	3	3	1	2	1	1	–	1	1	1	–	–	–	nt	2	3	–	–	–	–	–	–	–	–	–	–	–	1	1	–	–	–	–	2g	1e	–	–	–	–	–	–
"	115-M	2	2	2	2	–	2	3	3	1	2	1	–	1	1	1	1	–	–	–	–	2	2	–	–	–	–	–	3	3	3	3	4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
"	117-S	2	2	2	2	2	2	3	3	2	2	1	1	–	1	1	1	–	–	–	nt	2	2	–	–	–	–	–	1e	1e	1e	1e	–	–	–	–	–	–	1e	1e	–	1e	–	–	–	–	2e	–
Ps. aeruginosa	N.C.T.C. 5781	3	3	3	3	3	3	3	3	3	3	2	2	2	2	2	2	1	–	2	nt	3	3	–	–	–	–	–	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	IC (Alper)	3	3	2	3	4	3	4	4	3	3	2	2	2	2	3	2	2	–	2	nt	3	4	2	–	–	–	–	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	HCr. 13(")	3	3	3	3	3	3	4	4	3	3	2	2	2	3	3	2	1	–	2	nt	3	4	2	–	–	–	–	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	HCr. 5(")	3	3	3	3	3	3	4	4	3	3	2	2	2	3	3	2	1	–	3	nt	3	4	3r	3r	–	–	–	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	P.10(Fournier)	3	4	2	3	4	4	4	4	4	3	2	2	2	3	3	1	–	1	4	nt	4	4						4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

Explanatory –

1, 2, 3, 4 } extent of inhibition

e = partial inhibition

r = inhition occurring but dense growth on and in vicinity of primary streak

nt = not tested

– = resistant

Ps.pseudomallei strains as occasional trials with "weak" strains grown for prolonged periods (up to 1 week) showed stronger inhibitions on strains such as E.coli, Salmonella spp., etc. Two-three day cultures of a few strains of Ps.pseudomallei also exerted inhibitory effects on strains of Ps.aeruginosa. The only species which resisted the inhibitory effects of all strains of Ps.pseudomallei was Serratia marcescens. The inhibitory effects of 5 strains of Ps.pseudomallei (strains 35-SR, 19-R, 2-M, 92-S and 86-S) tested by the "streak" method against some of the strains belonging to other species are illustrated on Plates 38 and 39. It is proposed to refer to the inhibitory effect produced against other bacterial species as "alpha" effects to distinguish them from inhibitions caused by Ps.pseudomallei on strains within the species. Modifications to the testing procedure such as the growth of the "producer" strains on CAE or cellophane strips or the exposure of the growing producer strains to ultraviolet light for 2-3 minutes did not alter the inhibitory effects. Sterilisation of test plates prior to the application of the secondary streaks was also not found to cause any alteration in the inhibitory effects. The "alpha" effects were found to be similar to those caused by strains of Ps.aeruginosa on unrelated bacteria. Plates 40 and 41 illustrate the effects of Ps.aeruginosa (strain P.10) and of Ps.pseudomallei (strain 42-S) on an identical set of unrelated bacteria. The variations in the inhibitory effects appeared to be in intensity rather

than in the patterns of inhibition. Ps.aeruginosa caused these inhibitory effects much earlier in the course of incubation (24 hours) than Ps.pseudomallei which usually produced discernible inhibitions on or after 48 hours' incubation.

Many of the strains of Ps.pseudomallei also caused zones of inhibition on strains within the species. The most pronounced inhibitions were caused by the "mucoid" (M) dissociants. Non-induced cultures of such variants exerted inhibitory effects on all strains including themselves. The zones of inhibition caused by 24-hour cultures of "M" strains were nearly always uniform in extent and in intensity (Plate 42) though the occasional "M" strain tended to inhibit itself partially so as to leave a thinned growth. Frequently, the 48-hour cultures of the "M" strains were found causing more extensive inhibitions which resulted in the complete absence of growth on all secondary streaks. The inhibitory effects caused by the "M" cultures will be referred to as the "beta" reaction.

Much less pronounced inhibitions caused by a number of strains of Ps.pseudomallei have also been given in Table 14. These will be referred to as the "gamma" effects. They did not appear to be associated with any colony types since the "smooth" (S),"rough" (R) and "corrugated" (SR) type cultures, including those derived from primary strains giving rise to "M" dissociants, were found causing such effects. The producer strains showed specificities in not inhibiting

themselves and in inhibiting some but not all other strains within the species. However, the inhibitions were always weaker than those caused by the "M" strains, being limited usually to an area of 1-2 cm with the zones showing resistant colonies or thinned growth. Moreover, the inhibitions on some secondary streaks were interrupted since confluent and comparatively thick growths occurred on areas of such streaks lying over the primary streak (producer strain). Clear, 'punched-out' zones of inhibition comparable with those found in the "alpha" and "beta" inhibitions were never seen. Plate 43 illustrates the variations in inhibitory effects caused by the "SR" and the "M" dissociants of strain 38 (NCTC 8016). Both dissociants showed "alpha" inhibitions on the 3 unrelated strains (Past.haemolytica, B.subtilis and Staph.aureus) and neither caused any inhibitory effects on the strain of Ps.aeruginosa. The "M" dissociant partially inhibited itself and completely suppressed the growth of the "SR" dissociant of Ps.pseudomallei strain 38 as well as 7 other strains of Ps.pseudomallei. In contrast, the "SR" dissociant has shown no inhibitory activity towards itself, the "M" dissociant of strain 38 and towards 2 of the other 6 strains of Ps.pseudomallei. Its inhibitory effects even on the strains on which it has acted, have not been uniform. On 3 of these strains, the inhibitory effect has been so weak as to be hardly discernible. Ps.pseudomallei strain 15 appears to be the only one to be inhibited by the "SR" variant (38-SR) to a degree

comparable with that caused by the "M" variant (38-M).

Although "gamma" inhibitions were usually weak (Plates 44 and 45), more pronounced inhibitions were obtained on test-plates where some 'producer' strains had been subjected to UV-induction for 2-3 minutes (Plate 46). Often, however, many of the inhibited strains showed resistant growths on and adjacent to the site of the 'producer' streak (Plate 46). These producer strains causing interrupted inhibitions were those found to be lysogenic by Method 3. However, not all lysogenic strains were found causing interrupted inhibitions.

The "gamma" inhibitions were found to be less intense or completely suppressed on test-plates that had been exposed to heavy doses of UV-irradiation (exposure for 30 minutes) prior to cross-streaking with the strains under test for sensitivity. This was not observed on test-plates sterilised by exposure to chloroform vapour.

Since plate cultures (nutrient agar or tryptone soya agar) of the 'producer' strains showed a variety of inhibitory effects which did not appear to be associated with lysogeny, an attempt was made to identify the agents by differential centrifugation of fluids expressed from the plate cultures. For this purpose 8 of the strains (some lysogenic and some non-lysogenic) were grown on tryptone soya agar so as to permit the selection of known colony types which were each grown in 10 ml volumes of tryptone soya broth for 4 hours at 37°C. These cultures were used to seed sets of tryptone

soya agar plates of a standard thickness (8 mm). The excess fluid was removed and the plates dried and incubated at 37°C for 48 to 72 hours after which they were deep-frozen at -25°C for 4 to 5 hours. The plates were then thawed at room temperature and the culture liquors that extruded from the medium were collected and pooled. The pooled sample was centrifuged at 5000 G for ½ hour to sediment the cells, the supernatant fluid recovered and its pH measured after which it was sterilised by adding chloroform (5% v/v). Then the chloroform was removed and 120 ml of the fluid was centrifuged at 40,000 G for 3 hours in a refrigerated centrifuge. The supernatant fluid was retained and the sediment fraction suspended in normal saline, re-centrifuged and the sediment then re-suspended in 3 ml of saline (Fraction 1). The supernatant fraction was re-centrifuged at 120,000 G for 6 hours and the sediment washed once in normal saline and re-suspended in 3 ml of saline (Fraction 2). The supernatant fluid was retained as Fraction 3. Specimens of each fraction were prepared for electron microscopy. Aliquots of 1 ml from each fraction were subjected to tests for heat stability at 60°C and 75°C. The heated preparations along with the undiluted 'controls' were then assayed for activity by Method 3.

The pH of crude liquors of the various cultures are shown on Table 15. The liquors from the "mucoid" (M) type cultures generally gave higher pH readings than those from the other types. The activity of the fractions from various cultures are shown on Table 16.

TABLE 15

pH of crude liquors of eight Ps.pseudomallei cultures

Strain	lysogenic or non-lysogenic	pH of liquors from 48-hour TSA cultures	pH of liquors from 72-hour TSA cultures
15-M	l	8.4	8.4
28-SR	l	7.5	7.6
35-SR	l	7.3	7.6
44-M	l	8.6	8.5
46-S	l	7.4	7.5
55-S	nl	7.8	7.8
90-SR	nl	7.3	7.5
92-S	nl	7.4	7.6

TABLE 16

The activity of the various fractions (Fractions 1, 2 and 3) of the culture liquors of eight Ps.pseudomallei strains (grown on TSA for 48 hours) on some selected strains of Ps.pseudomallei and other bacteria

Strain	Lysogenic (L) or Non-Lysogenic (NL)	Fractions	Strains tested for sensitivity							
			Ps.pseudomallei				Ps.aeruginosa	Past.septica	Staph.aureus	Sarcina lutea
			2	4	27	83				
15-M	L	1	-	p	pp	-	-	-	-	-
15-M	L	2	-	p	-	-	-	-	-	-
15-M	L	3	-	-	-	-	-	bb	bb	bb
28-SR	L	1	pp	pp	pp	-	-	-	-	-
28-SR	L	2	-	p	-	-	-	-	-	-
28-SR	L	3	-	-	-	-	-	b	b	b
35-SR	L	1	-	-	b	pp	-	-	-	-
35-SR	L	2	-	-	-	-	-	-	-	-
35-SR	L	3	-	-	-	-	-	b	b	b
44-M	L	1	-	pp	p	-	-	-	-	-
44-M	L	2	-	p	-	-	-	-	-	-
44-M	L	3	-	-	-	-	-	bb	bb	bb
46-S	L	1	pp	pp	-	b	-	-	-	-
46-S	L	2	p	-	-	-	-	-	-	-
46-S	L	3	-	-	-	-	-	b	b	b
55-S	NL	1	-	-	-	-	-	-	-	-
55-S	NL	2	-	-	-	-	-	-	-	-
55-S	NL	3	-	-		-	b	bb	b	bb
90-SR	NL	1	-	-	-	-	-	-	-	-
90-SR	NL	2	-	-	-	-	-	-	-	-
90-SR	NL	3	-	-		-	-	b	b	b
92-S	NL	1	-	-	-	-	-	-	-	-
92-S	NL	2	-	-	-	-	-	-	-	-
92-S	NL	3	-	-		-	-	b	b	b

Explanatory: p = phage plaques
pp = zone of confluent inhibition but phage at dilution between 10^{-1} to 10^{-3}
b = weak zone of confluent inhibition with no visible plaques at higher dilutions
bb = strong to a moderate zone of inhibition with no visible plaques at higher dilutions.

Undiluted fractions 1 and 2 from the non-lysogenic cultures failed to show any inhibitory activity towards strains within the species and towards the other bacterial species. Fraction 3 preparations from these cultures showed varying degrees of activity towards some of the unrelated bacteria and no activity towards strains within the species. Undiluted Fraction 1 preparations from lysogenic cultures caused zones of clear, confluent inhibition on many of the Ps.pseudomallei strains tested for sensitivity and tests with serial dilutions of the preparations showed phage plaques or semi-confluent lysis at dilutions as low as 1 in 10. However, two of the Fraction 1 preparations (from strains 35 and 46) in addition to causing phage plaques or semi-confluent lysis with some indicators, caused weak bacteriocin-like inhibitions with other indicators (Plates 47 and 48). Undiluted Fraction 4 preparations from lysogenic cultures were found to be much less active. None showed any confluent zones of clear or opaque inhibition and only four showed phage activity. Neither Fraction 1 nor Fraction 2 preparations of the lysogenic cultures were found to exert any inhibitory effects or to cause phage plaques on the strains of Ps.aeruginosa or on strains of the unrelated bacteria. The Fraction 3 preparations from lysogenic cultures showed no activity on any of the strains of Ps.pseudomallei but like Fraction 3 preparations from non-lysogenic cultures, they showed varying degrees of inhibitory

activity towards the unrelated bacteria.

Heating at 60°C or 75°C for 1 hour was not found to destroy the inhibitory activity of the Fraction 3 preparations of either lysogenic or non-lysogenic cultures towards the unrelated bacteria. Heating at 60°C did not affect the phage activity of the Fraction 1 and 2 preparations of lysogenic cultures but heating at 75°C destroyed it. Heating at 75°C diminished the bacteriocin-like effects caused by the Fraction 1 preparations of strains 35 and 46 but did not destroy the effects completely.

Electron microscopy did not reveal phages or phage components in any of the Fractions from the non-lysogenic cultures. Except for the Fraction 1 preparations from strains 35 and 46, all lysogenic cultures also failed to show any phages or phage components. Even although the inhibitory or phage titres of the preparations from strains 35 and 46 were very low, they showed structures resembling the tails of bacteriophages. These tail-like structures were found in small numbers grouped together in a few areas of the specimen grid (Plate 49). A thorough search over entire grids did not reveal any intact phages or phage heads although faintly stained structures resembling empty phage heads were seen in close proximity to some of the tail-like structures (Plate 50).

(c) Ps.aeruginosa strains tested for antagonisms towards each other, towards Ps.pseudomallei and unrelated bacteria

The antagonistic effects of Ps.aeruginosa strains

towards unrelated bacteria were similar to those of Ps.pseudomallei and this was evident not only in tests using Method 2 ('lawn-on-spot') and Method 3 ('drop-on-lawn') but also in the 'streak' tests (see Table 14). The only differences observed were in the intensity of the inhibitory effects as was seen in tests using Ps.pseudomallei (strain 42) and Ps.aeruginosa (strain P10) against a set of unrelated bacteria (see Plates 40 and 41).

The antagonistic activity of the Ps.aeruginosa strains was not found to be limited to the unrelated bacterial strains. Ps.aeruginosa strains caused varying degrees of inhibition of Ps.pseudomallei strains when tested by the 'spot-on-lawn' method (Plates 51 and 52) and by the 'streak' method (Plate 53). The reactions seen in the latter tests were comparable with some of the reactions resulting from cross-tests of Ps.aeruginosa strains (Plate 54). Supernatant fluids from 24-48 hour nutrient broth cultures were not found to cause phage plaques nor any zone inhibitions on Ps.pseudomallei strains but supernates from 72-hour nutrient broth cultures caused varying degrees of inhibition on some strains. None of these inhibitions were found to be due to phage.

4. DISCUSSION

The application of techniques similar to those used in the detection of pyocinogenic strains of Ps.aeruginosa (Holloway, 1960; Wahba, 1963; Paterson, 1965; Osman, 1965; Gillies and Govan, 1969) proved useful in the investigations for inhibitory activity of Ps.pseudomallei towards strains within the species and towards strains of other species. In addition, the tests helped to demonstrate the antagonisms between Ps.pseudomallei and Ps.aeruginosa.

The tests using Methods 1, 2 and the 'streak' technique showed bacteriocin-like inhibitions caused by Ps.pseudomallei on strains within the species but a similar activity was not demonstrable by Method 3. However, this latter test revealed the high incidence of lysogeny in Ps.pseudomallei and indicated that many of the inhibition reactions to the other tests could be due to the lytic action of phages released by lysogenic strains (see Fig.4). The results of this test have emphasised the need to determine the lysogenicity of strains before interpreting inhibitions seen in the other biological tests (Method 1, 2 and 'streak') as due to "bacteriocins".

That the inhibitions seen in such tests (Methods 1, 2 and the 'streak' test) could also be due to agents other than bacteriocins and bacteriophages has been indicated by the "beta" reactions caused by "mucoid" (M) variants of Ps.pseudomallei on themselves and on

all other strains of the species. Bacteriocinogenic strains producing bacteriocins against themselves have been reported for Pasteurella (Brubaker and Surgalla, 1961), E.coli (Ryan, Fried and Mukai, 1955) and for Bacillus megaterium (Ivanovics, 1964) and a megacin with a wide action spectrum has also been described (Ivanovics, Alfoldi and Abraham, 1955), nevertheless, the high alkalinity found in "mucoid" (M) cultures of Ps.pseudomallei, the dialysability of the inhibitor (in such cultures) through cellophane ("Viskin") membranes known to allow only the passage of molecules less than 12,000 molecular weight, seem to rule out bacteriocins as the cause of the "beta" reactions. That "beta" inhibitions may be due to a factor other than a bacteriocin could be inferred from the findings of Rogul and Carr (1972). These workers using the 'streak' test of Wahba (1963) found their "smooth" strains of Ps.pseudomallei, in contrast to their "rough" strains, inhibiting all strains within the species including those producing the inhibitory agent. The "rough" strains were not found to exert any inhibitory effects upon any of the strains. These workers concluded that the inhibitory effect of the "smooth" strains was due to the production of ammonia which then accumulated in the agar medium to reach toxic concentrations. The "rough" strains, though found to produce ammonia, were also found to produce oxalic acid (a feature also observed by Nicholls, 1930) in amounts sufficient to neutralise most of the ammonia. Two of

the distinctive characters of the "smooth" strains described by Rogul and Carr (1972) were the loss of viability within a few days when grown on trypticase soya agar and the increasingly alkaline conditions produced in the medium as it became sterile. Thus, the characteristics of these "smooth" strains closely resemble those of the "mucoid" strains seen in this work (see p. 96 & 100). The "beta" inhibitions may therefore be due to ammonia toxicity.

The "gamma" inhibitions seen in the "streak" test, unlike the "beta" type, seemed to be due mainly to the lytic activity of phages since the majority of the strains causing this effect were those found to be lysogenic (as determined by Method 3) and the strains inhibited were usually those which, in Method 3, had shown sensitivity to the phages of these lysogenic strains. That many of these "gamma" reactions could be due to phage action of lysogenic strains could also be suspected from the size and intensity of the zone inhibitions since many of the zones were restricted to no more than 1-2 cm (suggesting a poorly diffusing agent) and since the inhibitions were partial (suggesting a possible lysogenisation of a proportion of cells in the "sensitive" cell populations). Many of the strains causing "gamma" inhibitions failed to do so on cellophane-laid test-plates which suggests the comparatively large particle size of the agent produced by such organisms. Many of the "gamma" inhibitions were also suppressed when test-plates prior to cross-streaking

were exposed to heavy doses of ultra-violet irradiation. Bacteriocins are considered to be proteins and are therefore unlikely to be inactivated by ultra-violet irradiation. The phages because of their nucleic acid make-up are more likely to be inactivated by ultra-violet irradiation. Ultra-violet induction of strains causing "gamma" inhibitions led to the production of more intense wide-zone inhibitions by some of the strains but they also allowed resistant growths of the "indicator" strains to occur on and in the vicinity of their own "streaks" (producer-streak). It would suggest lysogenisation of a proportion of "indicator" cells by phage from the "producer" strain rather than any cyclic production of a bacteriocin followed by a bacteriocin inhibitor, an explanation given to this phenomenon in Ps.aeruginosa by Wahba (1963).

That some of the "gamma" inhibitions may yet be due either in part or in whole to bacteriocinogenicity, can be considered from the finding of particles resembling phage components. Structures similar to these have been described as bacteriocins in some other bacterial species (Endo, Ayabe, Amano and Takeya, 1965; Mennigmann, 1965; Sandoval, Reilly and Tandler, 1965; Bradley, 1967, p.284; Coetzee, de Klerk, Coetzee and Smit, 1968) including Ps.aeruginosa (Kageyama and Egami, 1962; Homma, Goto and Shinoya, 1967; Ishii, Nishi and Egami, 1965; Higerd, Baechler and Berk, 1967, 1969; Govan, 1974a,b). Besides, some apparently non-lysogenic strains were found causing "gamma" inhibitions.

Modifications to the "streak" test (sterilisation of test-plates by heavy doses of ultra-violet irradiation prior to cross-streaking or the growth of the producer strains on cellophane-laid agar or CAE-agar) were not found to suppress the inhibitory effects of the non-lysogenic strains and such procedures, in some instances, were also not found to completely suppress the inhibitory effects of some of the known lysogenic strains even on "indicators" (as found by Method 3) of their phages. It would therefore seem that both bacteriophage and bacteriocin are involved in at least some of the "gamma" inhibitions. It may in part explain the reason for weak inhibitions. Interference between bacteriocin and bacteriophage production in some other bacteria has been observed by Frederiq (1955). Ps.aeruginosa strains simultaneously producing phages and bacteriocins active on the same "indicator" strains have been detected (Osman, 1965; Paterson, 1965; Shinoya, Goto, Tsukamoto and Homma, 1967; Rampling and Whitby, 1972). Some strains causing "gamma" inhibitions responded to ultra-violet induction by causing more pronounced inhibitions. A few of these strains were non-lysogenic. Thus it is possible that the effect of these non-lysogenic strains are due to the production of an inducible agent. In other bacteria, some bacteriocins, like some bacterio-phages, are known to be inducible by ultra-violet induction.

The inactivation of phages of Ps.pseudomallei by heavy doses of ultra-violet irradiation may be useful

in the detection of bacteriocins produced by strains which are both lysogenic and bacteriocinogenic or in differentiating bacteriocinogeny from lysogeny for typing purposes but there may be limitations. Ultra-violet inactivated phages of other bacteria have been known to be re-activated (Luria, 1947; Dulbecco, 1950; Harm, 1961) and, inactivated phages are known to attach to and kill host cells without replicating (Luria and Delbruck, 1942; Luria and Human, 1950; Cohen and Arbogast, 1950). Besides, the inactivating dose for different phages may vary considerably (Adams, 1966, p.65). Considering these aspects, it would seem unwise to rely on ultra-violet inactivation procedures to eliminate the phage activity of Ps.pseudomallei strains particularly in tests for bacteriocin activity. A further complication to the identification of strains on the basis of bacteriocin production or sensitivity may occur through the emergence of "mucoid" (M) dissociants in initially non-mucoid cultures. The study has shown this possibility. Their toxic alkaline metabolites that are probably the cause of the "beta" type inhibitions may alter the degree and the pattern of inhibition.

The present study as well as that of Rogul and Carr (1972) has failed to demonstrate bacteriocin-like substances in cultures grown in liquid media (nutrient broth or trypticase soya agar). In the present work, bacteriocin-like inhibitory effects of broth culture supernates of Ps.pseudomallei were only seen against

other species of Gram-negative or Gram-positive bacteria, a feature reported by Jameson (1949). Particular colony types were not found to be associated with these inhibitory effects though weak and strong 'producer' strains were evident. The inhibitory agent was not inducible nor was it found to be sedimentable by ultra-centrifugation. It was thermostable and dialysable. That this same agent could be the cause of "alpha" type inhibition is suggested by the similar properties found in the liquors expressed from the agar cultures. The agent, acting as it does on unrelated bacteria, cannot be considered a bacteriocin although Hamon (1956) reported the activity of a pyocin on E.coli and Cook, Blackford, Robbins and Parr (1964) found E.coli inhibiting Gram-positive bacteria.

The antagonistic activity of Ps.pseudomallei grown on solid media towards M.mallei, Ps.aeruginosa and towards several unrelated bacteria has been reported by Tomov (1970). Rogul and Carr (1972) also reported similar antagonisms towards Ps.cepacia and Ps.mallei but found no activity towards strains of Ps.aeruginosa (12 strains). Their failure to demonstrate antagonisms towards Ps.aeruginosa may be due to the choice of strains used since the present work has shown on the one hand, the occurrence of strains of Ps.pseudomallei that are weak or strong 'producers' of the inhibitor and, on the other hand, the occurrence of strains of Ps.aeruginosa that may not be inhibited even by the strong 'producers'.

The present work has also shown that bacteriocin-like inhibitions caused by Ps.aeruginosa on almost all strains of Ps.pseudomallei and the striking similarity of the two species in their antagonisms towards unrelated bacteria. The effect on unrelated bacteria may be due in part to similar inhibitors shared by the two species (pyocyanin not being the only antibiotic substance found in Ps.aeruginosa) which may point towards yet another relationship of the two species because of a common origin. If the antibacterial activity of Ps.pseudomallei towards unrelated bacteria be due to agents identifiable with the "classical" antibiotics, then it may also add support to the theory that the organism is a soil inhabitant since antibiotic producers such as members of the Bacillaceae and the Actinomycetales (Streptomyces, Microspora, Nocardia, etc.) are known soil inhabitants.

The demonstration of bacteriocin-like inhibitions between strains of Ps.aeruginosa and Ps.pseudomallei has drawn attention to the need, particularly in areas where melioidosis is endemic, for caution, especially in the pyocin typing of achromogenic strains of Ps.aeruginosa.

The results have shown that the identification of strains of Ps.pseudomallei or their differentiation from Ps.aeruginosa cannot be carried out on the basis of bacteriocin production or sensitivity mainly because other agents, both specific (bacteriophages) and non-specific (toxic alkaline metabolites) appear to be

involved in these inhibitory reactions. A follow-up of this study using physico-chemical methods to obtain purified preparations of bacteriocins may prove useful in typing work and in determining the nature of Ps.pseudomallei bacteriocins. It may establish whether the phage components seen in the present work are in fact a class of bacteriocins or are defective phages as shown for Colicine 15 (Endo et al., 1965; Frampton and Brinkley, 1965; Sandoval et al., 1965).

Part 4: Bacteriophages isolated from lysogenic strains of Ps.pseudomallei and their use in "phage typing"

1. INTRODUCTION

The occurrence of bacteriophages in Malleomyces spp. was first reported by Wolochow and Green (1956). In that same year, Leclerc and Sureau (1956) reported the isolation of 24 bacteriophages from water sources (lakes, ponds and rivers) in Vietnam that were found to be active on locally isolated strains of M.pseudomallei. By serial passage through these strains, they were able to obtain phage preparations capable of causing varying degrees of lysis (confluent lysis to discrete plaques) in 26 of 36 other strains of M.pseudomallei. These bacteriophages when tested on species of Achromobacter (25 strains), Flavobacterium (10 strains) and Ps.aeruginosa (8 strains) failed to lyse any of them. Serologically homogeneous strains of M.pseudomallei had shown differences in their susceptibility to these phages which suggested to these workers the existence of phage types within the same serological group. Differences in sensitivity to these phages were seen in some of the bacterial strains isolated from North and South Vietnam and this had led these workers to suggest the possible occurrence of antigenically different strains of M.pseudomallei in different regions.

Smith and Cherry (1957) examined 41 strains of M.pseudomallei and 23 strains of M.mallei for sensitivity to phages isolated from strains of M.pseudomallei. By using 16 of the strains of M.pseudomallei as 'indicators' they were able to isolate a total of 8 phages from some of the strains of this species. By passage of these

phages through cultures of M.mallei, they obtained 18 'adapted' phages. The original phages and in particular the 18 'adapted' phages were found to act more strongly on the cultures of M.mallei than on the cultures of M.pseudomallei. Four of the 'adapted' phages attacked 55 of the 64 cultures of Malleomyces spp. used in the study. None of the phages was found to act on 294 cultures of bacteria belonging to other species (including 49 cultures of Alkaligenes-like organisms and 24 cultures of pseudomonads). The generic specificity of the bacteriophages of M.pseudomallei led these workers to suggest that lysis by specific phages may prove useful in differentiating Malleomyces spp. from other closely related bacteria.

Jacobs, Dalton, Allison and Escobar (1972) examined a total of 48 strains of Ps.pseudomallei, originally isolated from patients in Vietnam, for bacteriophage activity. Of the 48 strains, approximately 40% were lysogenic and about 70% were found to be susceptible to the phages isolated, with at least 50% being susceptible to a single phage. Other species of the genus were not susceptible. On the basis of these findings, Dalton et al. thought that phages of Ps.pseudomallei may be useful in epidemiological studies as well as in the specific identification of the organism and/or in confirming laboratory diagnosis obtained by other means.

Since a large number of strains of Ps.pseudomallei examined for bacteriocinogenic activity (see:Part 3) were found to be lysogenic and the lytic spectra of some

of these lysogenic strains suggested the release of phage with different host ranges, it was considered desirable to isolate some of the phages for purposes of investigating whether (a) they could be used in identifying Ps.pseudomallei, (b) strains of Ps.pseudomallei could be differentiated on the basis of sensitivity patterns to selected phages, (c) phage types could be correlated to the possible occurrence of antigenic types and (d) phage typing could be used as an epidemiological 'marker'.

In the course of these investigations a few attempts were also made to detect whether changes in the production of haemolysis on blood agar could be brought about through an association between phage and its sensitive host and whether phage resistant 'mutants' emerging in phage-sensitive populations could differ from the parent strains in their in vitro sensitivity to antimicrobial agents.

2. MATERIALS AND METHODS

(a) Media

"Oxoid" nutrient agar or tryptone soya agar and nutrient broth or tryptone soya broth were used for the growth of Ps.pseudomallei strains employed in the isolation, purification and in the propagation of bacteriophages. In "phage typing" of Ps.pseudomallei strains, "Oxoid" nutrient agar was used in preference to tryptone soya agar.

Sheep blood agar plates were used to detect haemolysis resulting from an association between phage and its sensitive host.

The sensitivity of phage-resistant 'mutants' (picked from phage-sensitive populations) to antimicrobial agents was tested on plates prepared with "Oxoid" Diagnostic Sensitivity Test Agar.

(b) Incubation of cultures

All incubations were carried out at 37°C.

(c) Inducing agents and the procedure for inducing the production of phage

(i) Ultraviolet irradiation: The light source and the procedure used were similar to that described earlier for inducing the production of bacteriocins in liquid cultures except for the use of relatively young cultures (12-14 hour cultures) for exposure to the light source Preliminary trials using various exposure

periods (1 second, 4 seconds, 15 seconds, 30 seconds, 1 minute, 2 minutes, 3 or 4 minutes) indicated that broth culture suspensions exposed for periods between 1 and 2 minutes yielded phage more consistently than those exposed for longer or shorter periods. Induction was therefore carried out by exposing cultures for a period of 2 minutes. The cultures were then incubated for a further 6 hours before centrifuging to recover the supernatant fluid.

(ii) Mitomycin: Preliminary trials using 0.1 μg, 0.5 μg and 1.0 μg per ml of 12-14 hour nutrient broth cultures indicated that concentrations less than 1.0 μg/ml were ineffective in inducing the production of phage. The agent was therefore added to 12-14 hour cultures at the rate of 1.0 μg/ml of culture and cultures then incubated for a further 6 hours before recovering the supernatant fluid.

(d) Electron microscopy

The procedure was similar to that described for the study of bacteriocins. No attempts were made to concentrate phages by ultracentrifugation.

(e) Isolation of bacteriophages

From 74 (63.8%) strains found to be lysogenic (see: Fig.4, p.177) during the tests for bacteriocinogeny, 27 strains which showed differences in plaque morphology or in their lytic patterns against a battery of 10 "indicators" (strains 2, 4, 13, 15, 17, 27, 33, 74,79

& 83) were chosen for isolation of their phages.

The 27 lysogenic strains and the "indicators" were plated to nutrient agar and incubated for 48 hours to allow for colony differentiation. Material from a single colony of the predominant colonial type on each plate was inoculated on to an agar slope which was incubated overnight. These slopes were then used as working cultures. Where necessary, subcultures were prepared from them every 4-5 days using the plating technique as a means to pick out a colony morphologically similar to the one from which the working culture originated.

Except for minor modifications, the technique of isolating phage was similar to that used previously (see: Method 3, p.160) in attempts to differentiate bacteriocinogenicity from lysogenicity. Material from a slope culture of the lysogenic strain was inoculated into 10 ml of nutrient broth and incubated for 20 to 24 hours. It was then centrifuged at 4000 r.p.m. for 20 minutes and the supernatant fluid recovered and sterilised by adding chloroform (5% v/v). After removing the chloroform, a suitable volume of the supernatant fluid was diluted in nutrient broth to give 10-fold dilutions up to 10^{-5}. A standard drop (0.05 ml) of each of the preparations in the dilution series was spotted on to a set of 10 different "indicator" plates (each lawned with a different "indicator" strain by growing the strain in 10 ml nutrient broth for 5-6 hours, flooding 1-2 ml on to the surface of a

nutrient agar plate which, after pipetting off the excess fluid, was dried for 1 hour at 37°C with the lid partly open).

After spotting, the set of plates was dried for a further 10 to 15 minutes before incubating overnight. The plates were then examined for the presence of phage plaques on the spotted areas, the remainder of the lawn on each plate serving to control that the plaques were not due to a contaminant phage in the "indicator" culture. The "indicator" plate showing the highest number of plaques (whether of a clear or of an opaque type) was selected and a single plaque from it was picked for purification by passage through a fresh nutrient broth culture of the same "indicator".

Where a lysogenic culture was not found to yield plaques on any of the "indicator" plates, a further attempt was made using the ultraviolet- and mitomycin-induction procedures.

(f) Purification of phage

The centre of a selected phage plaque was touched with a straight wire and transferred to 2.5 ml of nutrient broth. To this was added 0.1 ml of a 6-7 hour nutrient broth culture of the appropriate susceptible strain (same strain as that from which the plaque was picked). The suspension was used to flood 2 or 3 nutrient agar plates which had been dried for not more than 1 hour at 37°C with lids partly tilted. After removing the excess fluid, the plates were dried for

a further 10-15 minutes and then incubated overnight. If discrete plaques were present, phage from a single plaque from any one such plate was picked for a further transfer using the same subculture procedure. The phage yield from this second transfer was harvested by scraping off the growth on each plate into 3 ml volumes of nutrient broth which were pooled and centrifuged at 4000 r.p.m. for 20 minutes. The supernatant fluid containing the purified phage was recovered and any residual bacteria in it were killed by adding chloroform (5% v/v). After removing the chloroform, the material was used for further propagation on the strain used for its purification.

(g) Propagation of phage

A method based upon that used by Williams Smith (1948) for the propagation of phages of staphylococci was employed.

(i) Broth method: 1 ml of the purified phage was added to 10 ml of a 6-7 hour nutrient broth culture of the propagating strain (i.e the strain used for purification). The culture was incubated overnight and then centrifuged at 4000 r.p.m. for 20 minutes to recover the supernatant fluid. A further passage in broth was carried out by adding 1 ml of this fluid to 10 ml of a 6-7 hour nutrient broth culture and incubating overnight. The supernatant fluid recovered by centrifugation of this culture was treated with chloroform (5% v/v) to kill any residual bacteria. After removing

the chloroform, the material was assayed for phage activity towards the propagating strain using the 'drop-on-lawn' assay procedure. If the phage activity in the undiluted lysate was found to be so low as to cause a few discrete plaques, the material was rejected and no further attempts were made to propagate the phage, but when the activity was high enough to cause semi-confluent to confluent lysis, the preparation was retained for further propagation of phage. This was carried out by the 'surface-plating' technique.

(ii) 'Surface-plating' method: Tryptone soya broth (10 ml) was inoculated from an agar slope culture of the propagating strain and incubated in a shaking waterbath (40 strokes/minute) for 6-7 hours. Equal volumes (approximately 3 ml) of the culture and the sterile broth culture lysate containing the phage were mixed and this phage/bacteria suspension used immediately for seeding 5 or 6 tryptone soya agar plates. Any excess fluid on the plates was pipetted off and the plates were dried for 1 hour at 37°C with their lids partly tilted. These were then incubated overnight. The growth from each plate was suspended in ca 3 ml of nutrient broth and the pooled suspensions centrifuged at 4000 r.p.m. for 20 minutes. The supernate was recovered and sterilised with chloroform. After removing the chloroform, a small volume was removed for assay to determine the phage titre and the "routine test dilution" (R.T.D.) while the bulk of the sample was held in the refrigerator (5°C). If the titre was

satisfactory, the preparation was stored as the "stock propagated phage" at 5°C since preliminary experiments showed that freezing to -25°C caused an 100- to 1000-fold loss of titre as compared with refrigerated samples which maintained their original titre for periods of up to 2 weeks, with about an 100-fold loss in titre in 1 month.

In addition to the chloroform method of preparing bacteriologically sterile propagated phage, other methods such as filtration through "Millipore" membrane filters (pore size: 0.22 μ) or heating to 58°C for 2 hours as used by Gould and McLeod (1960) in the preparation of Ps.aeruginosa phages were also tried using 5 ml aliquots of some of the centrifuged supernates.

(h) Designation of phages

Each propagated phage preparation is designated by a set of numerals. The system is similar to that used by Wilson and Atkinson (1945) for staphylococcal phages. The identification number of the lysogenic strain from which the phage was derived is placed first followed by the number of the strain on which it was purified and eventually propagated. Thus phage 75/2 would represent a phage derived from Ps.pseudomallei strain 75 and isolated, purified and propagated on Ps.pseudomallei strain 2 and likewise, phage 50/4 would represent a phage from Ps.pseudomallei strain 50, propagated on Ps.pseudomallei strain 4.

(i) Assay to determine the phage concentration and the "routine test dilution" (R.T.D.)

The 'drop-on-lawn' method basically similar to the technique employed in differentiating bacteriocin activity from phage activity (see: Method 3, p.169) was employed. Modifications were made with regard to the media used and the size of the standard drop of the phage preparation. The method was chosen in preference to the agar layer method (Adams, 1966, p.450) because of the considerable saving in time, plates and medium and because of the convenience in reading plaque counts and the R.T.D. from the same plate.

Method: The phage preparation was serially diluted in nutrient broth to give 10-fold dilutions up to 10^{-8}. Using a standard dropping pipette (0.025 ml/drop) and starting with the highest dilution, a drop each from the dilution series was placed on the test plate lawned with the appropriate propagating strain. The test plate was prepared by flooding the surface of a tryptone soya agar plate (9 cm diameter) poured to a standard depth (4-5 mm) with 1-2 ml of a 5-6 hour tryptone soya broth culture of the propagating strain and pipetting off the excess fluid. The plate after being dried for 1 hour at 37°C with its lid open, was spotted with the phage preparation and dried for a further 15 minutes. It was then incubated overnight. Phage concentration of the preparation was estimated by plaque counts. At this stage the preparations

yielding low phage titres and failing to produce confluent lysis except as undiluted material, were rejected. No further attempts were made to propagate these phages. Others were retained for testing against all Ps.pseudomallei strains. The highest dilution of each phage causing confluent lysis of its propagating strain was taken as the R.T.D. for "phage typing" of the various Ps.pseudomallei strains.

(j) Testing the sensitivity of various Ps.pseudomallei strains to the propagated phages

Strains representative of the 116 'primary' cultures (lyophilised cultures) in the collection were tested for sensitivity to the undiluted preparations of the various phages as well as against the preparations diluted to their R.T.D.

Working cultures (slope cultures) were prepared from platings of 'primary' cultures. These were used for periods up to 1 week. Before discarding them, fresh slopes were prepared from them using the plating technique previously described (p.209) to pick inocula from colonies morphologically similar to those used in the preparation of the original working cultures.

Test procedure: In the preliminary round of tests 13 phage preparations were used. Batches of 20-30 strains of Ps.pseudomallei were tested on any single day using the same batch of medium and stock phage diluted to R.T.D. Each strain to be tested was lawned on to two tryptone soya agar plates in the same way as for titrating

phage. Each phage preparation diluted to R.T.D. was spotted on to these plates (7 drops to a plate), which were then incubated overnight and readings taken the following day. (The R.T.D. of each phage preparation was checked 1-3 days prior to each batch of tests).

Subsequent tests were carried out using only 11 of the 13 phage preparations. The test procedure was similar to that used in the preliminary round except for the use of nutrient agar in place of tryptone soya agar. This change was made because the test strains grew more profusely on the TSA plates and allowed phage-resistant 'mutants' occurring within or around phage plaques to overgrow the plaques so rapidly as to obscure them.

After testing the 116 strains with the 11 phage preparations diluted to R.T.D., the strains were re-tested with the undiluted phage preparations.

The lytic reactions were scored as given below. Only the strong (+++) and moderate (++) reactions were considered in determining the lytic patterns.

strong = semi-confluent to confluent lysis including zones where the lysis amounted to an opaque clearing

moderate = approximately 50 or more plaques estimated roughly and not counted accurately

weak = less than 50 plaques

negative = no plaques

(k) <u>Test for the sensitivity of Ps.aeruginosa strains to phages of Ps.pseudomallei</u>

Six strains of <u>Ps.aeruginosa</u> were tested for

sensitivity to the 27 propagated phages. The phages were diluted to give five 10-fold dilutions and a drop of each dilution in a series was placed on a plate lawned with a 5-6 hour culture of the strain under test for sensitivity. The plates were incubated overnight and were examined for plaques.

3. RESULTS

The 27 lysogenic strains and the strains used for isolating and propagating their phages, and the maximum dilution of each propagated phage found causing confluent lysis or plaque forming units on its propagating strain, are given in Appendix D.

Isolation of phage: The 27 lysogenic strains caused phage plaques on one or more of the 'indicators' used, although the 'indicators' they attacked did not always correspond to those found in the previous study (see: Part 3, Fig.4). Many of the lysogenic strains caused turbid plaques or plaques with a resistant growth at the centre. Some produced clear and turbid plaques on the same 'indicator' or on different 'indicators'. Morphological variations were also found in the size of plaques produced on the same or different 'indicators'. Some phages in the course of purification produced turbid plaques (with resistant growth within plaques) varying in size (Plates 55 and 56) which were indicative of a lack of homogeneity of descendants even from single plaque subcultures.

Induction procedures were not found to be necessary except with 4 strains. These cultures induced by mitomycin gave lower yields compared with those obtained by ultraviolet irradiation.

Propagation of phage: Fourteen of the 27 propagated phages gave very low titres with confluent or semi-

confluent lysis occurring only in the undiluted preparations, and these were excluded from further study. Of the remaining 13 phages, 6 caused confluent lysis of their propagating strains up to dilutions of 10^{-4} or 10^{-5} and in these preparations the numbers of plaque-forming units, estimated from counts of plaques occurring at the higher dilutions, were found to be not less than 10^{8} particles/ml. The others gave relatively low yields, 4 preparations producing confluent lysis up to the 10^{-3} dilution and plaques up to the 10^{-6} dilution (approximately 10^{7} p.f.u./ml), 2 causing confluent lysis up to 10^{-2} and 1 up to 10^{-1}, although the phage concentrations in these last three preparations were found to be approximately the same. This lack of correlation between phage concentration and the maximum dilution causing confluent lysis appeared to be due to the size of the plaques. Phage preparations causing tiny plaques, whilst containing plaque-forming units to concentrations comparable with those causing large plaques, produced confluent lysis to relatively lower dilutions.

Culture supernates heated at 58°C for 2 hours to kill residual bacteria gave phage yields comparable with those treated with chloroform. On the other hand, "Millipore" filtration gave very low phage yields (no plaques to 10^{4} p.f.u./ml depending on the original titres). The latter result suggested adsorption of phage to the filter membrane.

Effect of ultraviolet irradiation for 2, 15 and

30 minutes: A simple assay procedure was used to determine whether the 13 phages which were all isolated from non-induced cultures, would withstand exposure to ultraviolet light for 2 minutes (the period found to be optimal for inducing phage production in most lysogenic cultures), for 15 minutes (the period within which 100% of the cell population of 5 bacterial cultures were found to be inactivated (see: Fig.1 p.[116])) and for 30 minutes (the period in which the inhibitory effects of cultures producing "bacteriocin-like" substances in "streak tests" seemed to be affected).

Each phage was diluted to R.T.D. in phosphate buffered saline pH 7.3 and 1.5 ml of each dispensed into disposable plastic petri plates and irradiated as previously described. Small volumes of these were drawn at the required intervals using "40-dropper" pipettes and spotted immediately to a test plate lawned with the appropriate propagating strain. A drop of non-irradiated phage was used on each plate as a control. The plates were incubated overnight and each spot compared with the control and examined for loss of confluent lysis. Confluent lysis was scored as 4-plus, semi-confluent lysis as 3-plus, approximately 25 or more plaques as 2-plus, less than 25 plaques as 1-plus and no plaques as negative. None of the phages was found to be affected by irradiation for 2 minutes. All produced only 2-plus or 3-plus reactions after irradiation for 15 minutes. Nine preparations showed no phage activity when irradiated for 30 minutes. The

other 4 phages (50/4; 75/2; 109/83 and 117/4) were also found to be affected considerably giving 1-plus or 2-plus reactions, indicating that they were also sensitive to prolonged exposure to UV light, though the rates of inactivation may differ. The assay procedure is unsuitable for making accurate estimates.

Electron microscopy: Phage particles were seen only in those preparations giving high yields of phage (10^8 p.f.u./ml or greater) and even in such preparations (phage strains 18/2; 28/4; 46/2; 75/2; 49/2; 116/33) the particles were not found in abundance. Often several fields had to be examined to see a single phage. Rarely a cluster of phages was seen. Three strains (28/4; 49/2 and 75/2) showed phages of a type having a head and a straight tail bearing the contractile appendage (Plate 57) although in some fields these intact phages were found amidst a small proportion of phage components (Plates 58 and 59). In some of these preparations the heads of intact phages appeared to be empty and the contractile appendage displaced towards the tail-end. Phages 18/2 and 46/2 also showed phage particles bearing tails, but the tail structures were not identical to those observed in the first three strains. Phage 18/2 showed particles with their tail sheaths uncoiled (Plates 60 and 61) and with a proportion of detached heads and tails. No tails or intact phages with coiled sheaths were seen during repeat tests. Phage 46/2 showed a particle with a relatively long curved tail bearing no sheath

either coiled or uncoiled (Plate 62). The material from the third preparation (phage 116/33) did not reveal tailed particles but only structures resembling phage heads, and these were not clearly discernible (Plate 63).

Little success was obtained in demonstrating the attachment of these phages to their host. Equal volumes of phage and dilutions of 4-5 hour broth cultures were mixed and examined within 5 to 10 minutes. One of 3 preparations of phages of the tailed type with coiled sheaths showed what appeared to be attachment to the host by means of the tail end without uncoiling the sheath (Plate 64). The other two preparations of this type of phage showed clusters of unattached phages (Plate 65). Examination of a specimen from the preparation that showed phages with uncoiled sheaths also showed attachment to the host by means of the tail. The sheath remained uncoiled (Plate 66).

Host range of the phages: The preliminary round of tests against the 116 strains of Ps.pseudomallei showed that the 13 phages (18/2; 28/4; 37/33; 46/2; 49/2; 50/4; 71/33; 75/2; 104/4; 108/2; 109/33; 116/33 and 117/4) had wide host range spectra. Three strains (28/4; 37/33; 71/33) had identical spectra and therefore only one (28/4) was included in the typing set. Some of the phages making the set shared properties in attacking some of the Ps.pseudomallei strains to similar degrees but showed differences in

their affinities towards other strains. The effects of the 11 phages used at R.T.D. on two different strains have been illustrated (Plates 67 and 68).

None of the 11 phages, even when used undiluted, was able to attack any of the 6 strains of Ps.aeruginosa (strains NCTC 7244; 5781; IC; HCR-5; HCR-13 and P.10). Any clearing due to a possible antibacterial activity in the phage suspension (as a result of harvesting off plate cultures) was not apparent.

Typing of Ps.pseudomallei strains on the basis of sensitivity to 11 selected phages: One or more of the 11 phages tested at their "routine test dilution " caused moderate to strong lysis of 74 (63.8%) of the 116 strains. One or more of these phages also caused weak reactions on a further 17 strains but none was found to attack the other 25 strains. Thirteen of the untypable strains had been isolated in Australia. On the basis of the strong and moderate reactions, 25 different sensitivity patterns (designated numerically) were recognised which, because of some of their similarities or dissimilarities, have been classified into 8 broad groups (Table 17). The sensitivity pattern of individual strains was not always reproducible and this, to some extent, appeared to be due to cultural variations occurring either in 'working' cultures or on the test plates. Evidence in support of this was gathered by testing different colony types from the same strain. For this purpose, test-plates were lawned

TABLE 17

Sensitivity groups and patterns shown by 74 strains of Pseudomallei pseudomallei to 11 phage strains diluted to R.T.D.

Group	Pattern No.	Susceptible strains of Ps.pseudomallei	Phage Strain No. 28/4	50/4	46/2	75/2	104/4	49/2	18/2	117/4	116/33	108/2	109/83
I-A	1	47;50;63;69;76;77;79	+										
II	2	36		+									
III	3	1;20;26;44;58;61;104;105;114;115			+								
IV	4	6;17;30;39;40;66;68;72;88				+							
V	5	100					+						
	6	none											
	7	none											
I-B	8	7;15;59;90;94	+		+								
	9	91;93	+		+			+					
	10	60;65;73;84;85;87;96	+				+						
	11	35	+	+									
	12	9;10;41	+	+			+						
	13	none											
	14	33;83	+	+	+	+	+				+		+
	15	3;38	+	+	+	+		+					
	16	none											
VI	17	13;27;29;107				+			+				
	18	70;117			+	+			+				
	19	none											
	20	none											
	21	16	+		+	+			+				
	22	none											
	23	67	+	+			+		+	+			
	24	none											
	25	78;99;116	+	+			+	+	+	+			
	26	62	+	+		+	+		+	+			
	27	95	+			+	+	+	+	+	+		
	28	none											
	29	19;24;34;48;81	+	+		+	+		+	+	+		
	30	none											
	31	111	+	+	+	+		+	+	+			
	32	none											
	33	112	+	+	+	+		+	+	+			+
	34	2	+		+	+		+	+	+	+	+	
	35	none											
	36	4	+	+	+	+	+	+	+	+			
	37	31		+		+		+	+	+			
	38	none											
	39	none											
	40	none											
	41	none											
VII	42	5						+					+
	43	none											
	44	none											

Broad groupings on the basis of patterns found related through action of certain phages (shaded columns) are shown as dotted cages.

with 5-6 hour nutrient broth cultures of selected colony types picked directly from nutrient agar plate cultures. The sensitivity patterns of 5 dissociants from 2 strains tested on the same day are given on Table 18. Patterns showed by different dissociants of the same strain have varied although such patterns have fallen into the same broad group.

When the undiluted phages were tested, they exerted stronger lytic effects and showed wider action spectra. As a result there were considerable alterations in the sensitivity patterns of some of the 74 strains reacting to the R.T.D. and a further 19 strains showing moderate or strong sensitivity reactions to one or more of the phages were added to the list. In all 32 patterns (which have also been designated numerically) were recognised by this test but again, many of the patterns showed relationships to warrant their inclusion under 9 broad groups (Table 19).

The various patterns of sensitivity (a total of 44 patterns) observed in the course of tests by the 2 methods (phages used at R.T.D. or undiluted) have been tabulated (Table 20).

Observations on phage-resistant colonies: It was observed that young broth cultures of propagating strains seldom cleared when propagated phage was added and overnight incubation gave rise to turbid growths comparable with untreated 'controls'. The "spot-on-lawn" assays carried out to determine the R.T.D. of

TABLE 18

The sensitivity patterns of 5 dissociants from 2 strains of Ps.pseudomallei.

Group	Susceptible strain of Ps.pseudomallei	Phage strains										
		28/4	50/4	46/2	75/2	104/4	49/2	18/2	117/4	116/33	108/2	109/83
VI	2-SR	+		+	+		+	+	+	+	+	
VI	2-S			+	+		+	+		+	+	
VI	2-M	+		+	+		+	+			+	
VI	4-SR	+	+			+		+	+			
VI	4-S	+	+	+	+	+	+	+	+			

TABLE 19

Sensitivity groups and patterns shown by 93 strains of Pseudomonas pseudomallei to 11 phage strains used undiluted

Group	Pattern No.	Susceptible strains of Ps.pseudomallei	Phage Strain No. 28/4	50/4	46/2	75/2	104/4	49/2	18/2	117/4	116/33	108/2	109/83
I-A	1	42;46;50;64;80	+										
II	2	36		+									
III	3	26;86;104			+								
IV	4	12;22;40;55;113				+							
	5	none											
IV-B	6	1;20			+	+							
IV-C	7	14;21;44;58;105;114;115		+	+	+						+	
I-B	8	7;15;59	+		+								
	9	93;94	+		+			+					
	10	60;63;65;69;76;77;79;84;85	+				+						
	11	35	+	+									
	12	9;10;41;47;66;87;89	+	+			+						
	13	90;91;92	+	+	+	+							
	14	none											
	15	none											
	16	100	+	+		+	+	+					
	17	107				+			+				
	18	none											
	19	13;27;39		+		+			+	+			
	20	6;17;29;30;68;70;72;88;117		+	+	+			+				
	21	16	+	+		+			+				
	22	3;38	+	+	+	+		+	+				
	23	none											
	24	67;73	+	+			+		+	+			
	25	none											
	26	none											
	27	none											
VI	28	57;62;78;95;96;99;116	+	+		+	+	+	+	+	+		
	29	19;24;34;81	+	+		+	+		+	+	+		
	30	48	+	+		+	+		+	+		+	
	31	none											
	32	111	+	+	+	+		+	+	+	+		
	33	none											
	34	2	+		+	+		+	+	+	+	+	
	35	31;112	+	+	+	+		+	+	+	+		+
	36	none											
	37	none											
	38	98		+	+	+		+	+	+	+		+
	39	83	+	+	+	+	+		+	+	+		+
	40	4;33;61	+	+	+	+	+	+	+	+	+		+
	41	75		+	+				+	+			
VII	42	5;25					+						+
VIII	43	8										+	
IX	44	23									+		

Broad groupings on the basis of patterns found related through action of certain phages (shaded columns) are shown as dotted cages.

TABLE 20

The different sensitivity patterns (44 patterns) recognised in the course of testing Ps.pseudomallei strains against the set of 11 phages used undiluted or diluted to R.T.D.

(Each pattern has been given a serial number)

Sensitivity pattern No.	Battery of phages										
	28/4	50/4	46/2	75/2	104/4	49/2	18/2	117/4	116/2	108/2	83/109
1	+										
2		+									
3			+								
4				+							
5					+						
6			+	+							
7		+	+	+						+	
8	+		+								
9	+		+			+					
10	+				+						
11	+	+									
12	+	+			+						
13	+	+	+	+							
14	+	+	+	+	+						
15	+	+	+	+		+					
16	+	+		+	+	+					
17				+			+				
18			+	+			+				
19		+		+			+				
20		+	+	+			+				
21	+	+		+			+				
22	+	+	+	+		+	+				
23	+	+			+		+	+			
24	+	+			+		+	+			
25	+	+			+	+	+	+			
26	+	+		+	+		+	+			
27	+			+	+	+	+	+	+		
28	+	+		+	+	+	+	+	+		
29	+	+		+	+		+	+	+		
30	+	+		+	+		+	+		+	
31	+	+	+	+		+	+	+			
32	+	+	+	+		+	+	+	+		
33	+	+	+	+		+	+	+	+		
34	+	+	+	+		+	+	+	+		+
35	+	+	+	+		+	+	+	+		
36	+	+	+	+	+	+	+				
37		+		+		+	+	+			
38		+	+	+		+	+	+	+		+
39	+	+	+	+	+		+	+	+		+
40	+	+	+	+	+	+	+	+	+		+
41		+	+				+	+			
42											+
43										+	
44									+		

phage preparations also showed the emergence of phage-resistant secondary growths or isolated colonies on areas of confluent lysis provided the plates were incubated for 48 hours or longer. This was thought to be due either to lysogenisation of a proportion of cells in the sensitive population by the added phage or to the selection of mutants naturally resistant to such phages. A very limited number of experiments was therefore carried out to determine whether the resistant growths were due to lysogenisation or otherwise and to determine whether such phage-resistant strains possess properties dissimilar to those of their phage-sensitive parent strains.

Procedures: 1 ml volume of propagated phage 49/2 preparation (containing approximately 10^6 p.f.u./ml) was mixed with an equal volume of a 1:100 dilution of a 5-6 hour culture of its propagating strain 2-SR. Similarly, phage 50/4 preparation (containing approximately 10^5 p.f.u./ml) and phage 109/33 preparation (containing 10^6 p.f.u./ml) were mixed with their respective propagating strains 4-SR and 33-S. Immediately after mixing, each of these phage/bacteria suspensions was flooded on to sheep blood agar plates that had been dried for 1 hour at 37°C with the lid tilted. The excess fluid was drawn off, the plate dried for a further 15 minutes and then incubated. By 48 hours well isolated colonies were found growing on all plates. The plate seeded with 2-SR + phage 49/2 showed colonies

that were morphologically similar to the parent 2-SR. For convenience, these phage-resistant colonies of 2-SR will be referred to as PR-2-SR. The plate seeded with 33-S + 109/33 gave rise to colonies of a "mucoid" type. These will be referred to as PR-33-M to differentiate from the sensitive parent 33-S. Like the cultures of the parent type, neither of these two phage resistant strains was found to produce haemolysis by 48 hours. Unlike the PR-2-SR and the PR-33-M type phage-resistant colonies, those arising from the plate seeded with 4-SR + phage 50/4 were not homogeneous and were of two types, neither showing resemblance to the 'SR' type parent. One (PR-4-R) was a "rough" (R) type similar to but not identical with those encountered as "R" type dissociants in Ps.pseudomallei cultures and the other was a slightly dwarfed and yellowish "rough" colony (PR-4-r) with a distinctly raised rough texture. After 48 hours' incubation, a clear haemolysis was present on this plate (Plate 69) although it was mostly around the colonies growing in close proximity to each other. However, it was noticeable that much of the haemolysis was due to the PR-4-r type colonies since the few found in isolation showed this feature.

When the phage-resistant colonies (PR-2-SR; PR-33-M; PR-4-R and PR-4-r) of the 3 cultures were subcultured to fresh blood agar, no changes were seen in those that arose from PR-2-SR and PR-33-M and phage plaques were not seen in the areas of continuous growth i.e. in the area of primary streaks. The plates of

PR-4-R and PR-4-r both showed a few scattered, discrete phage plaques in the area of continuous growth, and after 48 hours' incubation, it was apparent that the discrete colonies on plate PR-4-r were exclusively of the PR-4-R type (although on the areas of continuous growth, PR-4-r type colonies were visible). The colonies on plate PR-4-SR were homogenous and similar to the original PR-4-SR type colony. Both plates showed a weak haemolysis by 48 hours. When these plates were stored at room temperature, the haemolysis was found to be similar to that observed in most ageing cultures of Ps.pseudomallei (Plate 71). Suspensions of these colonies lawned on to plates showed scattered phage plaques. The occurrence of phage plaques on these plates indicated that the colonies were not lysogenised and that they are not naturally phage-resistant variants. Using the "replica-plating" technique of Lederberg and Lederberg (1952) the original plate (i.e. the plate seeded with 4SR + phage 50/4) which showed both PR-4-R and PR-4-r was replicated to a blood agar plate already seeded with a heavy dose of phage 50/4. The plate was incubated and by 48 hours, a marked haemolysis was seen around every colony (Plate 70).

Sterile supernates of nutrient broth cultures prepared from colonies of PR-2-SR and PR-33-M showed phage activity. The derived phages from PR-2-SR were found to attack Ps.pseudomallei strain 2-SR and the derived phage from PR-33-M was found to attack Ps.pseudomallei strain 33. A lawn of PR-2-SR spotted

with phage 49/2 was found not to be affected and similarly, a lawn of PR-33-M was found not to be attacked by phage 109/33. Neither of these lawns showed plaques on any other area of the plates. These features of PR-2-SR and PR-33-M were suggestive of lysogenisation. Changes in the antigenicity with PR-33-M have been dealt with elsewhere.

Antibiotic disc sensitivity tests carried out on these strains did not show patterns varying from those shown by the original parent strains (2-SR; 4-SR and 33-S). However, in a subsequent survey to detect the emergence of phage-resistance in other strains, it was found that the resistant colonies of a"mucoid" type emerging from Ps.pseudomallei strain 5 after exposure to a heavy dose of phage 49/2 varied from the parent strain in showing a higher degree of sensitivity to antibiotics (Plate 72). The sensitivity spectrum remained unchanged. The strain was not found to be lysogenised.

4. DISCUSSION

This work was initiated in the expectation that the phages isolated from selected lysogenic strains of Ps.pseudomallei might provide a means of differentiating the species from other pseudomonads such as the achromogenic strains of Ps.aeruginosa and, in addition, provide a means of recognising strains within the species.

Although the number of strains of Ps.aeruginosa examined for sensitivity to these phages is small, the findings have shown promise for the technique as a method of differentiating between the two species and have supported those of Leclerc and Sureau (1956) in this respect. The undiluted phage preparations used in this work showed no inhibitory effects on Ps.aeruginosa and may therefore have an advantage over preparations obtained by the "freezing and thawing" technique from agar cultures in which antibacterial agents inhibitory to some strains of Ps.aeruginosa may be present.

The use of a set of phages made up mainly of those propagated on one or other of two propagating strains (Ps.pseudomallei strains 2 and 4) must have limitations, since the ability to attack the same strain is an indication of a shared character however insignificant that may be in terms of the overall host range spectra of these phages It is therefore not surprising that the lytic patterns of the phages or the numerous phage sensitivity patterns (Table 21) of the various Ps.pseudomallei strains have shown less distinction and many overlaps. Phages with greater strain or group

specificities need to be found and it is possible that such phages are present in the existing collection since the numerous lysogenic strains (49.1%) showed differences in their patterns of sensitivity. It is well known that the sensitivity pattern of a strain is controlled, in part at least, by its own lysogenicity. Thus a number of more distinct phages may be present in the lysogenic strains which may have escaped detection because of the criteria used in isolating phages. 'Adaptation' procedures used for obtaining "typing" phages in other bacteria could have helped in finding phages of greater specificity but, since nearly all the Ps.pseudomallei strains investigated were found to be lysogenic, it was considered undesirable to experiment on these lines until completion of this preliminary investigation. 'Adapted' phages have been considered by some (Rountree, 1949) to be contaminant phage derived from the strain used for the propagation of the original phage.

Despite what may seem to be a set of phages lacking in group specificities, one or more of the 11 phages, used at R.T.D., attacked 74 strains of Ps.pseudomallei either moderately or strongly and 17 strains weakly. The phages, when used undiluted, attacked 93 strains either moderately or strongly and 6 strains weakly. The sensitivity patterns of the individual strains tested by these two methods have varied and variations in a number of instances, have affected the groupings (Tables 17 and 19). Although the patterns observed by

either method are an indication of the occurrence of strains, neither the patterns nor the groupings could be correlated to strains originating from different sources (Table 21). Unless other specific phages are found, the epidemiological 'finger-printing' might only be practicable in a given locality or country and not on a global basis.

The high rate of susceptibility to the 11 phages used undiluted could have been due mainly to "lysis from without" (see: Adams, 1966, p.173). The high percentage of strains reacting to the phages used at R.T.D. cannot be over-emphasised. Some of the strains received from the U.S.A., U.K. and France appear to be those obtained directly from laboratories in South East Asia and Australia and in all probability, many of them may be'duplicates' of stock laboratory cultures i.e. strains 65 and 84; strains 26 and 104. With the exception of those few isolated in Panama and Ecuador (which probably are also 'duplicates' i.e. 15 and 59; 55 and 92), other strains that have been received from the laboratories in Europe and the U.S.A. are those that had originated directly or indirectly (infected persons who had returned) from parts of South East Asia. Phage sensitivity patterns of some of the 'duplicates' may not be identical,which could be due to cultural variation or to differences through an acquired lysogeny. It is difficult to ignore the possibilities of accidental phage cross-contaminations occurring within laboratories handling these cultures.

TABLE 21

Relationship between country of origin of Pseudomonas pseudomallei and the phage sensitivity patterns and groups

Strain No.	Country of origin	Sensitivity pattern (group) undiluted phage	phage at R.T.D.
1	Sabah	6 (IV-B)	3 (III)
2	Sabah	34 (VI)	34 (VI)
3	Sabah	22 (VI)	15 (I-B)
4	Sabah	40 (VI)	36 (VI)
5	Sabah	42 (VII)	42 (VII)
6	Sabah	20 (VI)	4 (IV)
7	Vietnam	8 (I-B)	8 (I-B)
8	Vietnam	43 (VIII)	*
9	Malaysia	12 (I-B)	12 (I-B)
10	Malaysia	12 (I-B)	12 (I-B)
11	Thailand	*	*
12	Thailand	4 (IV)	*
13	Australia	19 (VI)	17 (VI)
14	Australia	7 (IV-C)	*
15	Ecuador	8 (I-B)	8 (I-B)
16	unknown	21 (VI)	21 (VI)
17	unknown	20 (VI)	4 (VI)
18	Australia	*	*
19	Australia	29 (VI)	29 (VI)
20	Australia	6 (IV-B)	3 (III)
21	Australia	7 (IV-C)	*
22	Australia	4 (IV)	*
23	Australia	44 (IX)	*
24	Australia	29 (VI)	29 (VI)
25	Australia	42 (VII)	*
26	Australia	3 (III)	3 (III)
27	Australia	19 (VI)	17 (VI)
28	U.K.	*	*
29	Sabah	20 (VI)	17 (VI)
30	Sabah	20 (VI)	4 (IV)
31	Sabah	35 (VI)	34 (VI)
32	Malaya	*	*
33	Singapore	40 (VI)	14 (I-B)
34	Singapore	29 (VI)	29 (VI)
35	U.K.	11 (I-B)	11 (I-B)
36	U.K.	2 (II)	2 (II)
37	U.K.	*	*
38	Australia	22 (VI)	15 (I-B)
39	Singapore	19 (VI)	4 (I-B)
40	Singapore	4 (IV)	4 (IV)
41	Malaya	12 (I-B)	12 (I-B)
42	Malaya	1 (I)	*
44	Malaya	7 (IV-C)	3 (III)
45	Malaya	*	*
46	Malaya	1 (I)	*
47	Malaya	12 (I-B)	1 (I)
48	Malaya	30 (VI)	29 (VI)
49	India	*	*
50	unknown	1 (I-A)	1 (I-A)
51	Malaya	*	*
52	Malaya	*	*
53	Dutch Antilles	*	*
54	Indonesia	*	*
55	Panama	4 (IV)	*
56	Malaya	*	*
57	Malaya	28 (VI)	*
58	Malaya	7 (IV-C)	3 (III)
59	Ecuador	8 (I-B)	8 (I-B)
60	Philippines	10 (I-B)	10 (I-B)
61	Malaya	40 (VI)	3 (III)
62	Malaya	28 (VI)	26 (VI)
63	Malaya	10 (I-B)	1 (I-A)
64	Malaya	1 (I-A)	*
65	Malaya	10 (I-B)	10 (I-B)
66	Malaya	12 (I-B)	4 (IV)
67	Malaya	24 (VI)	23 (VI)
68	Sabah	20 (VI)	4 (IV)
69	Malaya	10 (I-B)	1 (I-A)
70	Vietnam	20 (VI)	18 (VI)
71	N.Vietnam	*	*
72	N.Vietnam	20 (VI)	4 (IV)
73	Malaya	24 (VI)	10 (I-B)
74	Thailand	*	*
75	N.Vietnam	41 (VI)	*
76	Malaya	10 (I-B)	1 (I-A)
77	Malaya	10 (I-B)	1 (I-A)
78	Malaya	28 (VI)	25 (VI)
79	Malaya	10 (I-B)	1 (I-A)
80	Malaya	1 (I-A)	*
81	Malaya	29 (VI)	29 (VI)
83	Malaya	39 (VI)	14 (I-B)
84	Malaya	10 (I-B)	10 (I-B)
85	Malaya	10 (I-B)	10 (I-B)
86	Malaya	3 (III)	*
87	Malaya	12 (I-B)	10 (I-B)
88	Malaya	20 (VI)	4 (VI)
89	Malaya	12 (I-B)	*
90	New Guinea	13 (I-B)	8 (I-B)
91	New Guinea	13 (I-B)	9 (I-B)
92	Panama	13 (I-B)	*
93	unknown	9 (I-B)	9 (I-B)
94	unknown	9 (I-B)	8 (I-B)
95	unknown	28 (VI)	27 (VI)
96	unknown	28 (VI)	10 (I-B)
97	unknown	*	*
98	unknown	38 (VI)	*
99	Australia	28 (VI)	25 (VI)
100	Australia	16 (I-B)	5 (I-B)
101	Australia	*	*
102	Australia	*	*
103	Australia	*	*
104	Australia	3 (III)	3 (III)
105	Australia	7 (IV-C)	3 (III)
106	Australia	*	*
107	Australia	17 (VI)	17 (VI)
108	Australia	*	*
109	Australia	*	*
110	Australia	*	*
111	Sabah	32 (VI)	31 (VI)
112	Sabah	35 (VI)	33 (VI)
113	Sabah	4 (IV)	*
114	Sabah	7 (IV-C)	3 (III)
115	Sabah	7 (IV-C)	3 (III)
116	Sabah	28 (VI)	25 (VI)
117	Sabah	20 (VI)	18 (VI)
118	Indonesia	*	*

Explanatory:- * = Untypable

For example, laboratory equipment such as lyophilisation equipment may be effectively sterilised to prevent bacterial cross-contamination, but how effective this may be in destroying the free phage particles released by lysogenic bacteria is questionable.

Many of the strains appear to show multiple lysogeny. This is seen not only from the patterns of sensitivity of lysogenic strains but also from the differences seen in the lytic spectra of the lysogenic strains to those of the phages isolated from them. The lytic spectra of some of the lysogenic strains (see: Part 3, Fig.4) to those of the propagated phages derived from them (see: Tables 17 and 19) show the inability of some of the propagated phages to lyse those that their respective 'donors' were able to attack. It is therefore possible that multiple lysogeny is a common phenomenon in Ps.pseudomallei as it may be in Ps.aeruginosa (Holloway, 1960) and in the staphylococci (Rountree, 1949). The natural habitat of Ps.pseudomallei is considered by some to be rivers and swamps which may provide ample opportunity for different lysogenic strains of Ps.pseudomallei to pick up different phages without being lysed. Phage recombinants may arise. Strains multiply lysogenic or lysogenic to recombinant phages may occur in a given locality without the introduction of extraneous strains. Thus "phage typing" systems developed for Ps.pseudomallei for use even within a given locality may need to be revised from time to time.

From evidence gathered so far, it would seem that

phages of Ps.pseudomallei could be an effective aid in identifying the species. The phages used undiluted may have caused phage "lysis from without" but none of these preparations acted on the strains of Ps.aeruginosa. Of a total of 116 strains only 17 were not attacked by any phage, even when used undiluted, but further investigation may lead to the detection of phages acting both more specifically and also on those strains of Ps.pseudomallei which have shown resistance.

Inexpensive and readily available materials have been used in this work on phage typing. Methods are those than can be easily carried out in tropical regions where the disease is known to occur frequently. An assessment of the usefulness of phage typing of Ps.pseudomallei should be continued in these parts where workers will have access to freshly isolated strains.

The enhanced production of haemolysin through an association with phage requires further investigation. The haemolysis brought about by strain 4-SR in association with phage 50/4 did not appear to be due to phage-mediated transfer of a haemolytic factor from strain 50 since strain 50 itself did not possess a strongly haemolytic property. The phenomenon cannot be one of 'conversion' because material plated from a single colony of the resistant growth continued to show a varying number of plaques on growths occurring in serial subculture, indicating the persistence of the phage contaminant. The selection of a haemolytic

'mutant' already present in the population can also be ruled out because a single subculture resulted in the loss or in the lowering of the degree of haemolysis and moreover, the rough yellow dwarfed colonies which appeared to be the ones causing the haemolysis were not apparent. Their occurrence seems to be associated in some way with the continuous presence of excess phage particles of phage 50/4. It remains to be determined whether the association is one amounting to "pseudolysogeny" (Hayes, 1968). The increased sensitivity of the "mucoid" type variant derived from Ps.pseudomallei strain 5 after treatment with phage 49/2 also cannot be explained. These two features have not always been repeatable. Since the experiments required the use of a large volume of phage which was not prepared as a single batch, it is possible that the phages used were not identical strains but different phages carried by multiply lysogenic strains. Such an error could have been avoided had the phages been prepared in bulk.

In the electron microscopy studies on the morphological types of phages, difficulties were encountered in detecting them in several preparations because of low titres. Methods to concentrate phages were not attempted but such methods may have helped to reveal the morphology of phages in a wider sample. At least three morphological types of Ps.pseudomallei phage have been demonstrated and appear not to have been described previously. They appear to fit into

Bradley's (1967, p.236) classification of basic morphological types of bacteriophages. The tailed phage with the contractile appendage (Plate 57) resembles Bradley's Group A; the phage with the long curved tail lacking in a contractile appendage (Plate 62) may fit into Bradley's Group B and the tail-less phage (Plate 63) may fall into Bradley's Group D or E. The tailed phage 18/2 (Plate 61) which appeared to possess an uncoiled sheath could be considered as identical with the tailed variety possessing the contractile appendage since phages with the contactile appendage are known to uncoil it in the process of attachment to the host. In this work, free phage found in preparations of phage 18/2 were always found to have the tail appendage in the uncoiled state. Even though the procedures used in the preparation of phages were relatively mild and avoided those techniques such as ultra-centrifugation, freezing and thawing which may have been more damaging to the phage particles, phage components were still seen amongst the intact phages. Some of these components resemble structures that have been considered as large-particle "bacteriocins".

Of a total of 27 lysogenic strains, 14 gave low phage yields when propagated, even though the broth culture supernates of many of these strains caused confluent lysis on their 'indicators'. This suggests the release of more than one phage strain by a single lysogenic strain. Although all of these phages may attack the same 'indicator' some may be more virulent

than others. The propagation of different phages may require different culture conditions. The low yields of phage in these strains may also be due to the use of incorrect proportions of the phage/bacteria mixtures used for propagation on agar plates. Although doses sufficient to cause semi-confluent lysis were used, in some the phage particles may have been in excess and have caused "lysis from without".

Part 5: The antigenic characters of Pseudomonas pseudomallei

1. INTRODUCTION

Pseudomonas pseudomallei strains have been considered to be a serologically homogeneous group having antigenic relationships with some strains of Ps.mallei (Stanton and Fletcher, 1932; Cravitz and Miller, 1950a and b and Fournier, 1967). Legroux and Blanc (1943), in addition to pointing out a serological relationship with Ps.mallei, considered that Ps.pseudomallei may be related to Ps.aeruginosa since 31 strains of the latter tested against an anti-Ps.pseudomallei serum prepared in a sheep were found to cause agglutination to titres of 1 in 50 or over.

Brygoo (1959) found natural agglutinins to Ps.pseudomallei in some of 436 samples of human sera submitted for routine examinations. Agglutinins to Ps.pseudomallei were found to titres of 1 in 20 in samples from afebrile patients and to titres of 1 in 40 or over in samples from febrile patients especially those suspected of enteric fever. Fournier, Lajudie and Chambon (1953) also in the course of examining human sera found 4% false positive reactions to the agglutination test (formolized Ps.pseudomallei cell suspensions) in persons with Salmonella infections. They considered them to be due to a cross-relationship with Salmonella O antigens. Thomson (1933) and Beaver and Thomson (1933) have pointed to an antigenic relationship between Ps.pseudomallei and Actinobacillus

lignieresi.

Using the agglutination and agglutinin absorption tests, Chambon (1960) examined 96 strains of Ps.pseudomallei of human and animal origin from South East Asia and Australia and found that the somatic antigen was not homogeneous. He was able to demonstrate that, as well as a common antigenic component, there were fractions distinct to strains. On the basis of these differences, Chambon was able to classify all 96 of his strains into three groups consisting of 93, 2 and 1 strains respectively.

Laws (1967a and b) suspected the occurrence of antigenically distinct strains of Ps.pseudomallei in Australia since sera from culturally positive cases of melioidosis (sheep and man) had failed to react in haemagglutination-and complement-fixation tests against antigens prepared from heterologous strains.

Fournier (1965) in analysing the thermostable complex of Ps.pseudomallei found up to 6 precipitin lines in some of the strains but such findings had not permitted a classification into serologic types (Fournier, 1967). However, Dodin and Fournier (1970) were able to distinguish 2 serotypes by precipitin tests using agar-gel precipitation and immunoelectrophoresis methods. Of the 12 strains studied (6 from South East Asia and 6 from Australia) 9 (including 3 from Australia) were found to fall into 1 serotype and 3 others (all from Australia) were found to fall into a second serotype. Cross-agglutination tests

had helped to further differentiate 2 of the 3 strains in the second serotype, the third showing itself to be intermediate between the first and second serotypes.

A serological study was therefore undertaken to determine whether such differences could be found among the strains making up the present collection. In the course of the work a limited study was also made to find out the cross-reactivity of thermostable antigens of Ps.aeruginosa to anti-Ps.pseudomallei sera prepared in rabbits. An attempt was also made to investigate any changes in antigenicity that may occur in Ps.pseudomallei through acquired lysogeny.

2. MATERIALS AND METHODS

(a) Source of strains

The strains of Ps.pseudomallei and Ps.aeruginosa are those listed in Appendices A.1 and A.2 respectively.

(b) Media

Nutrient agar (Oxoid) dispensed as slopes in 4 oz. "medical flat" bottles was used for the preparation of the heated antigens and vaccines. Soft agar (nutrient agar containing 0.2% agar) was used for passaging Ps.pseudomallei for the selection of actively motile variants. Nutrient broth (Oxoid) was used for the preparation of formolised whole cell antigens and vaccines.

(c) Preparation of antigens

(i) Bacterial suspensions for the tube agglutination test. Each culture was plated to nutrient agar and incubated at 37°C for 2-3 days to allow for colony differentiation. A selected colony was used as follows:-

For the preparation of heated antigen (thermostable complex of the somatic antigen), the colony was inoculated into 10 ml of nutrient broth which was incubated for 4-5 hours and then used for seeding the surface of nutrient agar slopes. After withdrawing the excess inoculum, the slope cultures were incubated at 37°C for 24 hours. The growth was washed off in 2-3 ml of sterile saline (0.85% w/v), the yields of several bottles being pooled and centrifuged at 3000 r.p.m.

for 20 minutes. The supernatant fluid was discarded, the cell sediment resuspended in a fresh volume of saline and re-centrifuged at low speed. The cell sediment from this centrifugation was washed once more by repeating the cycle and cells finally suspended in a small volume (ca 5 ml) of saline and steamed at 100°C for 2 hours. The steamed suspension was adjusted in turbidity to give a scale reading of 2.5 on a portable colorimeter[1] using a green filter (OGR1). The aliquots withdrawn for turbidimetric readings were not returned to the adjusted suspension but were discarded. No preservative was added to the adjusted suspensions, which were held in the refrigerator for use as the Heated (Somatic) Antigen in the tube agglutination test.

For the preparation of formolised whole cell antigen, the selected colony type was passaged to obtain actively motile cells using a Craigie tube (Cruickshank, 1965, p.235) containing 0.2% agar but without any added serum. The agar in the inner tube was stab-inoculated to a depth of approximately three-quarters of the column and was then incubated at 37°C for 4-5 hours. Growth picked from the outer agar surface was inoculated into a 10 ml volume of nutrient broth and incubated at 37°C for 24 hours. The broth culture was centrifuged at 2000 r.p.m for 20 minutes, the supernatant discarded and the cell sediment washed once in normal saline before re-suspending in 5 ml of fresh saline. Formaldehyde was added to

[1] "EEL" Colorimeter, Evans Electroselenium Ltd., U.K.

a concentration of 0.04%. The suspension was then left to stand on the bench for 4-5 hours before storing at 4°C for 3-5 days. It was then diluted in saline to a density corresponding to the scale 2.5 on the colorimeter and then stored at 4°C for use as the Formolised Antigen.

(ii) Antigens for the production of antisera in rabbits. Heated cell suspensions of Ps.pseudomallei were used for the preparation of antisera against the thermostable antigenic complex. These suspensions are referred to as "heated vaccines" and were prepared in the same way as the Heated (Somatic) Antigen for the agglutination test except that the final turbidity adjustment was made to a scale reading of 4.0 on the colorimeter.

Formolised cell suspensions were used for the preparation of antisera against the whole cell antigens and are referred to as "formolised vaccines". The method of preparation was similar to that for Formolised Antigen except that adjustment of the final density of the suspension was made to a scale reading of 4.0 on the colorimeter. The formolised vaccine was checked for sterility by inoculating approx. 0.1 ml into 10 ml of nutrient broth which was incubated at 37°C for 3 days.

(iii) Antigens for immuno-diffusion and immuno-electrophoresis. The growth on 2 nutrient agar slopes was suspended in about 10 ml of saline and washed

twice in saline by centrifugation. The supernatant fluid was removed and the sediment suspended in approximately 10 ml of distilled water. The fixed suspension was then alternately frozen (-20°C) and thawed 10 times and then centrifuged at 4000 r.p.m. for 20 minutes. The supernatant fluid was recovered for use as the "crude aqueous extract" (CAE antigen) in the precipitation tests. The material was stored at -20°C.

(d) Preparation of sera

Eleven sera were prepared in rabbits against the heated and formolised vaccines of Ps.pseudomallei strains 1, 8, 13, 14, 23, 27, 74, 102, 108, 111 and 112.

Prior to immunisation, the rabbits were bled to test for natural antibodies to the immunising strains. Rabbits received 7 intravenous injections of vaccine at intervals of 4 days, the first, second and subsequent doses being 0.25 ml, 0.5 ml and 1 ml respectively. Injections were made into the marginal vein of one ear, the other ear being reserved for bleeding by vene puncture. Test-bleedings were made 7 days after the last injection and if the agglutinating titre was satisfactory, further bleedings were made from the ear and the animal finally bled out by cardiac puncture. If the titre proved unsatisfactory, two further injections were given and a test bleeding made after an interval of one week.

Antisera were preserved by adding merthiolate to a concentration of 0.01%.

(e) Absorbed sera

Absorption of antibodies against the thermostable antigenic complex from antisera prepared against formolised vaccines, was carried out. The bacterial strain used for production of the antiserum was grown on nutrient agar slopes, the growth from each of two slopes suspended in 3-4 ml of sterile saline and the whole made up to 10 ml with saline. The suspension was centrifuged and the supernatant discarded. The washing by centrifugation was repeated twice more and, after the third centrifugation and removal of the supernatant fluid, the packed cells were steamed (100°C) for 2 hours. After cooling, 5 ml of a 1 in 5 dilution in saline of the antiserum to be absorbed was added to the packed cells and mixed. After incubation at 37°C for 2 hours and overnight storage at 4°C, the mixture was centrifuged to recover the absorbed serum. A tube agglutination test was then carried out against the heated antigen of the absorbing strain to determine whether the absorption had been complete. Usually a single absorption was enough to remove the antibodies against the thermostable complex but, as a routine, a second absorption was always carried out.

A similar procedure was adopted in absorbing the antibodies against the shared thermostable antigens

in the antisera produced against heated vaccines. The heterologous strain sharing the antigen was grown on nutrient agar slopes. It was harvested, washed, heated as described above, and mixed with the diluted antiserum. After incubation at 37°C for 2 hours and refrigeration overnight, the absorbed serum was recovered by centrifugation. As before a single absorption was found to be adequate to remove the antibodies against the shared antigen, but as a routine, a second absorption was carried out.

(f) Tube agglutination tests

Tube agglutination tests were carried out in 50 mm x 6 mm test-tubes (Durham tubes) and incubated for 18-20 hours in a waterbath at 56°C. Serial doubling dilutions of serum were made in phenol saline (0.5% phenol) from 1 in 10 upwards, the range covered being determined by the titre of the serum for its homologous antigen. An equal volume of the antigen was added to each tube. A spring-loaded syringe was used in the preparation of the serum dilutions and addition of the antigens.

(g) Immuno-diffusion

One per cent "Ionagar" No.2 (Oxoid) in barbitone acetate buffer, ionic strength 0.1, pH 8.6 with 0.01% merthiolate was used. Two ml volumes of the melted preparation were layered on to 76 mm x 25 mm microscope slides on a levelled platform and allowed to set. A gel cutter (cutting a central well and

six peripheral wells, each 4 mm diameter) was used for cutting micro-wells. The bottom of each well was then sealed by placing a drop of the molten ionagar so as to prevent any seepage of antiserum or antigens from the respective wells. The wells were then filled using small bore pipettes, the antiserum filling the central well and the various antigens filling the peripheral wells. The slides were then placed in large square petri plates lined with moistened filter paper and incubated for 2-3 hours at 37°C before placing in a cold room at 4°C. They were examined for precipitin bands at 24 and 48 hours. After that, they were stained, examined and photographed where necessary.

(h) Immuno-electrophoresis

One per cent "Ionagar" No. 2 (Oxoid) in barbitone acetate buffer, ionic strength 0.1, pH 8.6 with 0.01% merthiolate was used in 5 ml volumes to give a level layer on clear glass plates (8 cm x 8 cm). Wells (1 mm diameter) and troughs (1 mm width) were cut so that the distance between 2 wells on either side of a trough was approximately 6 mm. The wells were filled with the antigens and the plate placed in an electrophoresis[1] tank and electrophoresed for 2 hours at 50 volts. Following this the agar band in each trough was removed and the troughs filled with antiserum. The plate was stored at 4°C to allow the development of precipitin bands. Staining, when

[1] Shandon Scientific Company, United Kingdom.

carried out was similar to that described below.

(i) Staining technique to clarify precipitin bands

The technique was similar to that described by Cruickshank (1965, p.952). Any unprecipitated material was washed out of the agar by immersion of the slide or plate for 24 hours in the buffer used for preparing the agar gel. It was then washed for 15 minutes in 1% acetic acid and placed in a staining trough containing a 1% solution of naphthalene black in glacial acetic acid/distilled water/methylated spirit solvent (1:49:50). After overnight staining the slide was washed with the solvent until the precipitin bands could be seen against a clear background. It was then soaked in 1% acetic acid containing 1% glycerol until differentiation was complete. A moistened filter paper cut to a suitable size was then placed evenly on the surface of the gel layer on the plate or slide to cover the whole surface and the slide was placed in the incubator at 37^{o}C to dry overnight. The plate or slide was then examined for precipitin bands.

3. RESULTS

Initially three strains (Nos. 74, 111 and 112) were selected for antiserum production. These were chosen as representing two recently isolated strains derived from animals and a soil strain. Each serum was tested against all 116 strains of Ps.pseudomallei, both heated and formolised antigens. In general terms the formolised antigens were agglutinated to the same titre as the homologous strains by all three sera prepared against formolised vaccines. When antisera prepared with heated vaccines were used to test all strains of the collection, all were agglutinated but ten (Nos. 1, 8, 13, 14, 21, 23, 27, 102, 108 and 109) were agglutinated only to low titres. The remainder were agglutinated to the same titre as the homologous strains. Eight of the ten strains showing low titres were selected for further antiserum production.

Tube agglutination tests

(a) Tests with antisera to formolised vaccine absorbed with the heated antigen of the homologous strain: All 116 strains of Ps.pseudomallei were tested against the eleven absorbed antisera and in all cases titres ranging from 1 in 1280 to 1 in 5120 were obtained. In every case the difference between the result with the homologous strain and other strains was never more than one dilution tube. The formolised cell preparations of six strains (Nos. 19, 24, 32, 51, 61 and 85) were not tested since they showed auto-

agglutination. Reciprocal cross-absorption tests with eleven strains (Nos. 1, 8, 13, 14, 23, 27, 74, 102, 108, 111 and 112) and their sera showed no differences, each strain absorbing the antibody to the homologous strain.

(b) Tests with antisera prepared against heated vaccines: The eleven antisera, with the exception of those against strains nos. 1 and 8, caused complete agglutination to a titre of 1 in 1280 when tested against the homologous strain. Of the antisera, five (Nos. 1, 8, 74, 111 and 112) agglutinated 108 strains to titres comparable with those observed against the homologous strains. The titres against the remaining 8 strains (Nos. 13, 14, 21, 23, 27, 102, 108 and 109) were low and the agglutination amounted to a partial clearing (approximately 25%). Such variations were not observed when the antisera prepared against some of these strains (Nos. 13, 14, 23, 27, 102 and 108) were tested against heterologous strains including strains nos. 1, 8, 74, 111 and 112. The results are summarised in Table 22.

(c) Tests with antisera to heated vaccines after cross-absorption with heated antigen of heterologous strains: The results are given in Tables 23a, 23b, 23c and 23d. From these it can be seen that strains nos. 8, 74, 111 and 112 (Group I) all behave similarly and their absorbed antisera all give similar results. This is the same for strains

TABLE 22

Agglutination titres of antisera to heated antigens

Anti-serum against strain no.	Reciprocal titre to heated antigen of strain no.													
	74	111	112	1	8	13	14	21	23	27	102	108	109	All Other Strains
74	1280	1280	1280	640	1280	80	80	80	80	80	80	80	80	1280
111	1280	1280	1280	640	1280	80	80	80	80	80	80	80	80	1280
112	1280	1280	1280	640	1280	40	80	80	80	80	160	160	160	1280
1	640	640	640	640	640	80	80	80	80	80	160	160	160	640
8	640	640	640	640	640	40	40	40	40	40	40	40	40	640
13	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280
14	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280
23	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280
27	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280
102	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280
108	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280

TABLE 23a

Agglutination tests with absorbed sera. Antisera 8, 74, 111 or 112 absorbed with heated antigens of heterologous strains

Serum absorbed with antigen	Reciprocal agglutination titre against strain no.													
	13	14	21	102	108	109	23	27	1	8	74	111	112	All other strains
13	-	-	-	-	-	-	-	-	40	80	80	80	80	40-80
14	-	-	-	-	-	-	-	-	40	80	80	80	80	40-80
102	-	-	-	-	-	-	-	-	40	80	80	80	80	40-80
108	-	-	-	-	-	-	-	-	80	80	80	40	80	40-80
23	-	-	-	-	-	-	-	-	10	40	40	40	40	40
27	-	-	-	-	-	-	-	-	10	40	40	40	40	40
1	-	-	-	-	-	-	-	-	-	10	10	10	10	10

TABLE 23b

Agglutination tests with absorbed sera. Antisera 23 or 27 absorbed with heated antigens of heterologous strains

Serum absorbed with antigen	Reciprocal agglutination titre against strain no.													
	13	14	21	102	108	109	23	27	1	8	74	111	112	All Other strains
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-
102	-	-	-	-	-	-	-	-	-	-	-	-	-	-
108	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	40	40	40	40	40	40	20	20	-	-	-	-	-	-
8	40	40	40	40	40	40	40	40	-	-	-	-	-	-
74	40	40	40	40	40	40	40	40	-	-	-	-	-	-
111	40	40	40	40	40	40	20	20	-	-	-	-	-	-
112	40	40	40	40	40	40	20	20	-	-	-	-	-	-

TABLE 23c

Agglutination tests with absorbed sera. Antisera 13 or 14 absorbed with heated antigen of heterologous strains

Serum absorbed with antigen	Reciprocal agglutination titre against strain no.													
	13	14	21	102	108	109	23	27	1	8	74	111	112	All Other strains
102	-	-	-	-	-	-	-	-	-	-	-	-	-	-
108	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	10	10	10	10	10	10	-	-	-	-	-	-	-	-
27	10	10	10	10	10	10	-	-	-	-	-	-	-	-
1	80	80	80	160	160	160	80	80	-	-	-	-	-	-
8	160	160	160	160	160	160	80	80	-	-	-	-	-	-
74	160	160	160	160	160	160	80	40	-	-	-	-	-	-
111	80	80	80	80	80	80	40	40	-	-	-	-	-	-
112	80	80	80	80	80	80	40	40	-	-	-	-	-	-

TABLE 23d

Agglutination tests with absorbed sera. Antisera 102 or 108 absorbed with heated antigen of heterologous strains

Serum absorbed with antigen	Reciprocal agglutination titre against strain no.													
	13	14	21	102	108	109	23	27	1	8	74	111	112	All Other strains
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	10	10	10	10	10	10	-	-	-	-	-	-	-	-
27	10	10	10	10	10	10	-	-	-	-	-	-	-	-
1	40	40	40	40	40	40	40	40	-	-	-	-	-	-
8	80	160	80	80	80	80	40	40	-	-	-	-	-	-
74	80	80	80	80	80	80	40	40	-	-	-	-	-	-
111	80	80	80	80	80	80	40	40	-	-	-	-	-	-
112	80	80	80	80	80	80	40	40	-	-	-	-	-	-

23 and 27, 13 and 14, and 102 and 108 Thus there appear to be four groups separable as a result of these absorption tests although the second, third and fourth of these show considerable overlaps. It is therefore possibly more satisfactory to regard these as subgroups of a major group. Therefore all strains of Ps.pseudomallei in the collection may be grouped in Group I with the exception of strains nos. 1, 13, 14, 21, 23, 27, 102, 108 and 109. These latter may be regarded as falling into a second group (Group II) which has two subgroups:-

Group IIa comprising strains nos. 1, 23 and 27

Group IIb comprising strains nos. 13, 14, 21, 102, 108 and 109.

Immuno-diffusion tests

Tests were carried out using all 11 antisera prepared with heated vaccines with antigens derived from the homologous strains and showed that a component in the thermostable antigen complex was shared by all strains of Ps.pseudomallei and this component was recognisable by a precipitin band (band closest to the central well) appearing as a reaction of identity when several strains were tested simultaneously. However, the strains found classifiable into groups on the basis of reactions to the agglutinin-absorption tests, showed their group characters (Group I, Group IIa or Group IIb) by producing an additional precipitin band unique to each group. Plate 73 shows that strains nos. 112, 74, 111 and 8 of Group I

tested against antiserum 112 have each produced two precipitin bands whereas strain 102 (belonging to Group IIb) has produced only a single band which shows a reaction of identity with the inner band produced by the Group I strains. It was found that the antibody fraction reacting with Group I strains to produce the inner band, could be absorbed by any one of 8 strains (nos. 13, 14, 21, 23, 27, 102, 108 and 109). Such an absorption is illustrated on Plate 74. Group I strains when tested against antisera 13, 14, 23, 27, 102 or 108 produced only a single band and such reactions are illustrated on Plate 75 where strain 111 has been tested alongside strains 102, 108, 14, 23 and 27 against antiserum 102. The band produced by strain 111 shows a reaction of identity with the innermost band produced by each of the other 5 strains. The same Plate shows clearly that strains 23 and 27 (both isolated in Australia) are not identical with the Australian strains 102, 108 and 14 since their outermost precipitin bands do not show reactions of identity. That strains 23 and 27 are themselves not identical to each other can be seen from the reaction of partial identity (intersecting bands) produced when they are tested against antiserum 23 (Plate 76). The antiserum 23 used for this demonstration was a sample that had been previously absorbed with strain 102 and therefore it is possible to see that the absorption has not removed a factor in serum 23 reactive to strains 14 and 108. Reactions similar to that shown by 14 and 108 were also observed

when the sample of serum 23 was tested against strains 21 and 109. Serum 14 absorbed with strain 102 presented a similar picture (Plate 77) though in this instance strain 27 gave a very faint precipitin band. From this analysis, it would appear that strains isolated elsewhere than in Australia are a homogeneous group even in relation to thermostable precipitinogens and as in the agglutination tests, they have fallen into a single group. The strains from Australia which fell into Group I in the agglutination tests also showed no deviations and they all cross-reacted with the sera produced against the strains isolated elsewhere than in Australia (strains nos. 74, 111 and 112) to give precipitin bands identical with those seen against the homologous strains. The other Australian strains, (strains nos 13, 14, 21, 23, 27, 102, 108 and 109), although they showed a distinctive group character in their agglutination and precipitin reactions in relation to all other strains, showed some differences amongst themselves. Strains 13, 14, 21, 108 and 109 seemed to be identical in their precipitin reactions. Strains 102, 23 and 27 seemed to be subgroups within this group.

Variations in precipitation reactions due to the use of different dissociants were not observed. Although eight of the strains isolated in Australia from man or domestic animals fell into one of two broad groups in agglutination or precipitation tests and some among the eight strains showed differences in precipitin components, these were not found to be related to phage-

sensitivity patterns except with strains 14 and 21. These two strains were identical in their precipitin reactions and in their sensitivity patterns to the set of typing phages (see: Part 4). Strains 13, 23 and 27 differed from each other and from strains 14 and 21 in their sensitivity to the phages. Strains 102, 108 and 109 were among those found to be resistant to all phages, propagated or otherwise. Since strains 14, 21, 108 and 109 were found to be lysogenic and broth culture supernates of these strains were found to contain phage active on the non-lysogenic strain 33, a preliminary investigation was carried out to determine whether the phage-resistant colonies arising in cultures of strain 33 treated with these four phages were different from the parent strain in their antigenic components. Attempts to obtain lysogenised colonies from strains 14, 21 and 108 were not successful but, as mentioned in Part 4, a lysogenised strain (strain PR-33-M) was obtained from strain 33 by treatment with propagated phage 109/33. Immuno-diffusion tests carried out with a preparation of strain PR-33-M against antiserum 111 showed a single, somewhat diffused inner band whereas the parent strain showed a double band. Tested against antiserum 108, strain PR-33-M gave slightly diffused precipitin bands, corresponding to those produced by strains 108 and 109 but dissimilar to the single band produced by strain 33 (Plate 78).

Immuno-electrophoresis

The method did not provide adequate separation of

the components and this was most evident in tests of the various strains with sera prepared against strains 8, 74, 111 and 112. Not more than 2 precipitation bands were observed against strains that fell into Group I in the agglutinin-absorption tests and a single band was always observed against the other strains. Such bands observed against serum 111 are shown on Plate 79. The antiserum produced against strain 1 (which fell into Group IIa), produced more discernible lines against the various strains. It produced 3 bands against the homologous strain and against the Australian strains 13, 14, 21, 27, 102, 108 and 109 and usually one or two bands against the other strains. The reactions observed against some of the strains have been illustrated (Plate 80). The antisera 23, 27, 13, 14, 102 and 108 also produced two bands against the strains that fell into Group I in the agglutination test. Serum 23 produced three bands against strain 1 while the other 5 sera produced two bands against this strain. Antiserum 23 also showed a difference from the other 5 sera in producing two bands against strain 102 while producing three bands against strains 13, 14, 21, 27, 108 and 109. The reactions of antiserum 23 to some of these strains are illustrated (Plate 81). The action of antiserum 27 was similar to that of antiserum 23 except that it produced three bands against strain 23.

Antisera 13, 14, 102 and 108 also produced two or three bands against strain 1 and two or three bands against strain 23 although these antisera produced six

bands against each of 7 Australian strains (nos. 13, 14, 21, 27, 102, 108 and 109). The reactions of some of the strains against antiserum 108 are illustrated in Plate 82.

Agglutination tests against Ps.aeruginosa strains.

Antisera prepared against the formolised and heated suspensions of strains of Ps.pseudomallei partially agglutinated all 6 strains of Ps.aeruginosa only at a dilution of 1 in 10. Agglutinin-absorption tests were not carried out.

4. DISCUSSION

Stanton and Fletcher (1932), Cravitz and Miller (1950) and Fournier (1967) in detecting antigenic relationships between Ps.pseudomallei and Ps.mallei found no evidence of serological heterogeneity in Ps.pseudomallei although Fournier (1967) noted that antiserum against one of the Ps.pseudomallei strains used in the study gave a low agglutinating titre against the other 6 strains and vice versa. It is perhaps the findings of these workers that have led to the assumption that strains of Ps.pseudomallei form a serologically homogeneous group.

It would seem that the failure on the part of the earlier investigators to detect antigenic differences was due largely to the use of small samples of strains collected from endemic regions within countries in South East Asia, since Chambon (1960), using 96 strains collected from South East Asia and Australia, was able to classify the strains into 3 groups on the basis of differences in the O complex. The number of strains that fell into his second and third groups (1 and 2 strains respectively) may seem small but it cannot be considered insignificant since, more recently, a finding similar to that of Chambon (1960) has been reported by Dodin and Fournier (1970). These workers used the gel diffusion, immuno-electrophoresis and cross-agglutination tests to analyse the antigenic components of the thermostable complex (considered to

be their O and K antigens) and the thermolabile complex (considered as H and M antigens) of 12 strains (6 from South East Asia and 6 from Australia). They distinguished two serotypes (I and II) by using the immunodiffusion and immunoelectrophoresis tests on the thermostable antigenic complex. Strains which made up Group II were limited to three of the 6 Australian strains. They considered the Group II strains to be rare outside Australia. By means of cross-agglutination tests these workers found it possible to separate two of the three strains in Serotype II from the third which appeared to behave as an intermediate between Serotypes I and II.

The present investigation has to a large extent supported the findings of Chambon (1960). Although all strains in the present collection have shown themselves to share at least a thermostable antigenic component, it has been possible, by means of agglutination tests with absorbed sera, to distinguish two serological groups, Group I made up of 107 strains including some from Australia and Group IIb made up of six Australian strains and another Group IIa made up of strains 23 and 27 from Australia and strain 1 from Sabah.

The immuno-diffusion tests have confirmed beyond doubt the existence of two serologically distinct groups in relation to thermostable antigens, but on the basis of this test, it would seem more likely that two strains (nos. 23 and 27) of the three in

Group IIa are more closely related to, although not identical with, strains in Group IIb and that strain 1 has similarities with those in Group I. These findings are in general agreement with those of Dodin and Fournier (1970). Three of the strains used by these workers (PA, T8+8 and J53) were employed in the present investigation (strains nos. 13, 14 and 23 respectively). Dodin and Fournier found that some Australian strains differed from Asian strains in having one precipitin band missing. The present work has not borne this out since both Australian and Asian strains have yielded two bands (one of which was common in strains from the two sources). The findings in the present study indicate that all strains possess a common precipitinogen to give a reaction of identity (see: Plates 73 and 75; band closest to central well) but that each group contains one other component characteristic of the Group (see: Plate 73 where strain 102 is found to lack a precipitin band common to strains in Group I, and Plate 75 where strain 111 is found to lack a band common to strains in Group II). The immuno-diffusion study has also revealed that strain 23 (Fournier's strain J53) and strain 27, although differing from strains in Group I and showing a closer relationship to strains in Group II do not show reactions of identity with those Group IIb strains but that strain 27 tested against serum 23 shows a reaction of partial identity with strain 23 (i.e. Group IIa strains).

Fournier (1965) using the Ouchterlony technique studied 12 strains of Ps.pseudomallei isolated from human beings and found variable lines of precipitation for the same serum depending upon the batch of antigen and for the same antigen depending upon the batch of serum. Repeating the experiments with strong sera, he was able to show a maximum of 6 precipitin lines. Though differences in precipitin lines were observed when a serum was tested against heterologous strains, they were not found to be sufficiently reproducible to warrant a serological classification. In the present study, the Vietnam strains along with all strains from South East Asia were found to produce no more than 2 precipitin bands even though different batches of antigen were used against the same serum. It is possible that the constituents of Fournier's thermostable antigen (which he has referred to as OK to include somatic and capsular antigens) may have brought about these additional precipitin bands. The thermostable antigens used in the present study were those prepared cell harvests that had been washed thrice which may have removed extracellular materials.

In immuno-electrophoresis tests it was possible to recognise 1 or 2 precipitin lines in strains that had been classified in Group I (see:Plates 79 and 80) and up to 6 lines in strains classified in Group IIb (see: Plate 82), but again it was clear that strains 23 and 27 when reacting with sera of strains belonging to Group IIb showed 2 or 3 lines. The strains of

Group IIb tested against sera of strains 27 and 23 (see: Plate 81) showed 3 lines.

In clinical investigations the haemagglutination test has been considered to be more specific and sensitive than the agglutination test in the diagnosis of melioidosis (Fournier, Lajudie and Chambon, 1955; Ileri, 1965; Strauss et al., 1967). However, Laws (1967) found a false-negative reaction to the haemagglutination test in a proven case of melioidosis. In the haemagglutination test red blood cells coated with the thermostable antigenic complex are used. Should the sensitivity in the haemagglutination test be dependent, in part or in whole, on the antibody factor to the unshared thermostable components of Ps.pseudomallei strains, then there is justification for the use of a polyvalent antigen from precipitin groups such as those detected in the present study.

Levine and Maurer (1958) isolated an avirulent mutant from a virulent strain of Ps.pseudomallei by ultraviolet irradiation. By repeated inoculation into mice, they observed a significant immune response to parenteral challenge with 10^3 or 10^4 cells of the parent strain and two other virulent strains, although the protection against two further strains used at similar doses was found to be much less. The origins of these strains have not been described but the differences in protection may reflect antigenic differences. The protective antigens may reside in the unshared antigens.

Phage conversion of serotypes have been reported for Salmonellae (Uetake, Nakagawa and Akiba, 1955; Uetake, Luria and Burrows, 1958), Vibrio foetus (Ogg and Chang, 1972) and for Ps.aeruginosa (Liu, 1969). The findings with regard to the changes in the thermostable antigens in PR-33-M appear to be due to a lysogenic conversion. The differences in antigenic structure brought about by phage conversion may have important implications in serotyping. Since all strains of Ps.pseudomallei have been found to be serologically homogeneous in tests with antisera prepared against formolised antigens, the test may have its merits in identifying strains, but the auto-agglutination in saline of suspensions of rough (R) strains have indicated the limitations of this test.

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APPENDIX A.1: Sources of strains of Ps.pseudomallei

Strain No.	Sender, source and further references
1.	Animal Disease Research Centre, Sabah, Malaysia. Ref No. VL411/69 isolated from a lung abscess of a pig in June 1969.
2.	Animal Disease Research Centre, Sabah, Malaysia. Ref. No. VL459/69 isolated from a lung abscess of a sheep in 1969.
3.	Animal Disease Research Centre, Sabah, Malaysia. Ref. No. VL463/69 isolated from a lung abscess of a pig in 1969.
4.	Animal Disease Research Centre, Sabah, Malaysia. Ref. No. VL524/69 isolated from a lung abscess of a goat in 1969.
5.	Animal Disease Research Centre, Sabah, Malaysia. Ref. No. VL493/69 isolated from a spleen abscess of a goat in 1969.
6.	Animal Disease Research Centre, Sabah, Malaysia. Ref. No. 577/69 isolated from the heart blood of a rabbit in 1969.
7.	Dr. J. Fournier, Pasteur Institute, Paris. Ref. No. 6068 isolated from a human patient in Vietnam.
8.	Dr. J. Fournier, Pasteur Institute, Paris. Ref. "Dalat" isolated from a human in Vietnam.
9.	Dr. J. Fournier, Pasteur Institute, Paris. Ref. "USAMRU-1" isolated from soil in Malaysia.
10.	Dr. J. Fournier, Pasteur Institute, Paris. Ref. "USAMRU" isolated from soil in Malaysia.

Strain No.	Sender, source and further references
11.	Dr. J. Fournier, Pasteur Institute, Paris. Ref. "UBOL-1" isolated from soil in Thailand.
12.	Dr. J. Fournier, Pasteur Institute, Paris. Ref. "UBOL-2" isolated from soil in Thailand.
13.	Dr. J. Fournier, Pasteur Institute, Paris. Ref. "PA" isolated from a human in Australia.
14.	Dr. J. Fournier, Pasteur Institute, Paris. Ref."T8+8"isolated from a goat in Australia.
15.	Dr. J. Fournier, Pasteur Institute, Paris. Ref. No. 4889 isolated from a human in Equador.
16.	Royal (Dick) School of Veterinary Studies, Edinburgh. A laboratory strain in use at the Veterinary school for a number of years. Origin not known.
17.	Royal (Dick) School of Veterinary Studies, Edinburgh. Another laboratory strain in use at the Veterinary school. Origin not known.
18.	Dr. M. Laws, Animal Research Institute, Yeerongpilly, Queensland, Australia. Ref. No. G1983 isolated from a sheep.
19.	Dr. M. Laws, Animal Research Institute, Yeerongpilly, Queensland, Australia. Ref. "O" isolated from a human.
20.	Dr. M. Laws, Animal Research Institute, Yeerongpilly, Queensland, Australia. Ref. No. C119 isolated from a horse.
21.	Dr. M. Laws, Animal Research Institute, Yeerongpilly, Queensland, Australia. Ref. No. T878 isolated from a goat.

Strain No.	Sender, source and further references
22.	Dr. M. Laws, Animal Research Institute, Yeerongpilly, Queensland, Australia. Ref. No. W2815 isolated from an ox.
23.	Dr. M. Laws, Animal Research Institute, Yeerongpilly, Queensland, Australia. Ref. No. J.53 isolated from a sheep.
24.	Dr. M. Laws, Animal Research Institute, Yeerongpilly, Queensland, Australia. Ref. "O" isolated from a goat in Australia.
25.	Dr. M. Laws, Animal Research Institute, Yeerongpilly, Queensland, Australia. Ref. "5-Water" isolated from water.
26.	Dr. M. Laws, Animal Research Institute, Yeerongpilly, Queensland, Australia. Ref. "Searle" isolated from a human.
27.	Dr. M. Laws, Animal Research Institute, Yeerongpilly, Queensland, Australia. Ref. "123-man" isolated from a human.
28.	Mr. K.J. Stokes, Pathology Department, St. James' Hospital, Balham, London, U.K. Ref. NCTC A52/68 isolated from a sternal abscess from a human - Pakistani immigrant admitted as a patient to the hospital. (See: Stokes, K.J. and Sylvia McCarthy (1969) J. med. Lab. Technol. 26: 199).
29.	Animal Disease Research Centre, Sabah, Malaysia. Ref. No. VL622/69 isolated from the spleen of a rabbit.
30.	Animal Disease Research Centre, Sabah, Malaysia. Ref. No. VL646/69 isolated from the spleen of an orang-utan at the Orang-utan Rehabilitation Centre, Sabah.
31.	Animal Disease Research Centre, Sabah, Malaysia. Ref. No. VL225/70 isolated from a goat lung in 1970.

Strain No.	Sender, source and further references
32.	National Collection of Type Cultures No. 1688, isolated from a rat in 1923 by W. Fletcher in Malaya. See: Stanton, A.T. and Fletcher, W. (1925). J. Hyg. Camb. 23, 347 and Brindle, C.S. and Cowan, S.T. (1951) J. Path. Bact. 63: 471.
33.	National Collection of Type Cultures No. 4845, isolated by W. Young, Singapore in 1935 from a natural infection in a laboratory monkey. See: Brindle, C.S. and Cowan, S.T. (1951) J. Path. Bact. 63: 471 and Lysenko, O. (1961) J. gen. Microbiol. 25: 379.
34.	National Collection of Type Cultures No. 4846, isolated by W. Young, Singapore in 1935 from a natural infection in a laboratory monkey. See: Brindle, C.S. and Cowan, S.T. (1951) J. Path. Bact. 63: 471.
35.	National Collection of Type Cultures No. 6700, isolated from a human patient in 1942. See: Grant, A. and Barwell, C. (1943) Lancet 1: 199 and Brindle, C.S. and Cowan, S.T. (1951) J. Path. Bact. 63: 471.
36.	National Collection of Type Cultures No. 7383, isolated from a West African soldier in 1946 who has been serving in Burma. See: Harries et al. (1948) Lancet 1: 363 and Brindle, C.S. and Cowan, S.T. (1951) J. Path. Bact. 63: 471.
37.	National Collection of Type Cultures No. 7431, isolated from a human at the Guy's Hospital, London. See: Jameson, J.E. (1949) J. Hyg. Camb. 47: 142 and Brindle, C.S. and Cowan, S.T. (1951) J. Path. Bact. 63: 471.

Strain No.	Sender, source and further references
38.	National Collection of Type Cultures No. 8016, isolated from a sheep in Australia in 1949. See: Brindle, C.S. and Cowan, S T. (1951) J. Path. Bact. 63: 471.
39.	National Collection of Type Cultures No. 8707-"Bendler A.", isolated in 1946 in a SEAC laboratory in Singapore.
40.	National Collection of Type Cultures No. 8708-"Bendler B.", isolated in Singapore in 1946.
41.	National Collection of Type Cultures No. 10274, isolated from a human case in Kuala Lumpur, Malaya. See: Montgomery, R. (1963) J. Royal Army Med. Corps 109: 223 and Hart, R.J.C. and Bradshaw, R.P. (1962) Trans. (R.) Soc. trop. Med. Hyg. 56: 265.
42.	National Collection of Type Cultures No. 10276 isolated from a human from an abscess over the 8th rib in 1962 in Malaya. See: Maegraith, B.G. and Leithead, C.S. (1964) Lancet, 1: 862.
43.	Dr. Lim Teoh Wah, Institute for Medical Research, Kuala Lumpur, Malaya. Ref. "Yakob" (IMR-KL, Malaya). Isolated from a human.
44.	Dr. Lim Teoh Wah, Institute for Medical Research, Kuala Lumpur, Malaya. Ref. "Mueng" (IMR-KL, Malaya). Isolated from a human.
45.	Dr. Lim Teoh Wah, Institute for Medical Research, Kuala Lumpur, Malaya. Ref. "Davies" (IMR-KL, Malaya). Isolated from a human.

Strain No.	Sender, source and further references
46.	Dr. Lim Teoh Wah, Institute for Medical Research, Kuala Lumpur, Malaya. Ref. "Hontana" (IMR-KL, Malaya). Isolated from a human.
47.	Dr. Lim Teoh Wah, Institute for Medical Research, Kuala Lumpur, Malaya. Ref. "Mai" (IMR-KL, Malaya). Isolated from a human.
48.	Dr. Lim Teoh Wah, Institute for Medical Research, Kuala Lumpur, Malaya. Ref. "Meru" (IMR-KL, Malaya). Isolated from soil.
49.	Dr. G.A. Hottle, Naval Biological Laboratory, University of California. Strain NBL.O-102(2). Strain MP-F of Wetmore and Gochenour (1956). Originally received from the Army Medical Service Grad. School, Washington as strain 295 of the Calcutta School of Tropical Medicine.
50.	Dr. G.A. Hottle, Naval Biological Laboratory, University of California. Ref. strain NBL.O-104(2). Strain MP-H of Wetmore and Gochenour (1956). Originally received from the Army Medical Service Grad. School, Washington, as strain "China 3".
51.	Dr. G.A. Hottle, Naval Biological Laboratory, University of California. Ref. strain NBL 111-1. Received in 1953 from the Institute of Medical Research, Kuala Lumpur, Malaya as strain Wong Fook Mook. Isolated from human infection.
52.	Dr. G.A. Hottle, Naval Biological Laboratory, University of California. Ref. strain NBL 113(2). Received from the Institute of Medical Research, Kuala Lumpur, Malaya as strain "Horse (IMR)", isolated between 1947-49 from an equine infection in Malaya.

Strain No.	Sender, source and further references
53.	Dr. G.A. Hottle, Naval Biological Laboratory, University of California. Ref. strain NBL 124. Originally received in 1957 from Institut voor bacteriologie, Rijksuniversiteit, Utrecht, Netherlands as Aruba strain II. Isolated in 1955 from an infected sheep in Netherland Antilles. Ref. Sutmoller, Kraneveld and Schaaf (1957).
54.	Dr. G.A. Hottle, Naval Biological Laboratory, University of California. Ref. strain NBL 125. Originally received in 1957 from Institut voor bacteriologie, Rijksuniversiteit, Utrecht, Netherlands. Isolated from human infection in Indonesia.
55.	Dr. G.A. Hottle, Naval Biological Laboratory, University of California. Ref. strain NBL 128. Originally received from Division of Veterinary Medicine, Walter Reed Army Institute of Research as Strain M-1444. Isolated 1957 from human infection in Panama.
56.	Dr. G.A. Hottle, Naval Biological Laboratory, University of California. Ref. strain NBL 130. Originally received from Microbiol. Division, Fort Detrick, U.S.A. as strain P.W. 124/61. Isolated 1951 from infected pig in Malaya.
57.	Dr. G.A. Hottle, Naval Biological Laboratory, University of California. Ref. strain NBL 133. Originally received from Microbiol. Division, Fort Detrick, U.S.A. as strain P.W. 140/60. Isolated 1960 from infected horse in Malaya.
58.	Dr. G.A. Hottle, Naval Biological Laboratory, University of California. Ref. strain NBL 135. Originally received from Microbiol. Division, Fort Detrick, U.S.A. as strain P.W. 62/61. Isolated 1961 from an infected goat in Malaya.

Strain No.	Sender, source and further references
59.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. 4889. Isolated from a human in Equador. See: Biegeleison et al., Am.J.Trop. Med. & Hyg. 1964, 13: 89.
60.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref.MP-D. Isolated from a horse in the Phillipines. Originally received from Dr. Cornel, Bureau of Animal Industries, Republic of the Phillipines in 1961.
61.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. MP-E. Isolated from a horse in Malaya. See: Brit. Med. J. 1952, 108: 161.
62.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Isolated from a horse in Malaya. Originally received from the United States Army Medical Research Unit, Kuala lumpur, Malaya in 1964.
63.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. MAP-31. Isolated from surface water in Malaya. Originally received from the United States Army Medical Research Unit, Kuala Lumpur, Malaya on 24.2.64.
64.	Dr. A.D. Alexander, Department of Veterinary Microbiology, Walter Reed Army Institute for Research. Ref. MAP-144. Isolated from surface water in Malaya. Originally received from the United States Army Medical Research Unit, Kuala Lumpur, Malaya on 6.8.64.

Strain No.	Sender, source and further references
65.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. MAP-174. Isolated from a parrot in Malaya. Originally received from the United States Army Medical Research Unit, Kuala Lumpur, Malaya on 6.8.64.
66.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. MAP-175. Isolated from a goat in Malaysia. Originally received from the United States Army Medical Research Unit, Kuala Lumpur, Malaya on 6.8.64.
67.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. MAP-501. Isolated from a monkey at a Zoo. See: Strauss, et al., Am.J.Vet.Research, 1969, 155: 1169
68.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. MAP-505. Isolated from an Orang utan in Sabah, Malaysia. Originally received from the United States Army Research Unit, Kuala Lumpur, Malaya on 20.12.70.
69.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. MAP-502. Isolated from a human case in Malaya. Received from the U.S. Army Medical Research Unit, Kuala Lumpur, Malaya in December, 1965.
70.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. 7820. Isolated from the infected finger of a United States soldier in Vietnam in 1966.

Strain No.	Sender, source and further references
71.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. 7919. Isolated from the sputum of a United States soldier who had served in North Vietnam. Originally received in March, 1966.
72.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. 7947. Isolated from a fatal case of a United States solider who had served in North Vietnam in April, 1966.
73.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. 5314. Isolated from a pig in Malaysia. Originally received from the United States Army Medical Research Institute, Kuala Lumpur, Malaya in October, 1963.
74.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. 7181. Isolated from the surface water in Thailand. Originally received from the United States Army component, SEATO Laboratory, Bangkok, Thailand on 3rd May, 1965.
75.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. 8675. Isolated from the urine of a soldier who served in North Vietnam. Originally received in August, 1969.
76.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 168/55. Isolated from a goat in Malaya in October, 1955.

Strain No.	Sender, source and further references
77.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 187/55. Isolated from a goat in Malaya on 7th December, 1955.
78.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. "Indra 1965". Isolated from a horse in Malaya in 1965.
79.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 71/61. Isolated from a pig in Malaya on 30th March, 1961.
80.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 230/61. Isolated from a wild goat in Malaya on 24th March, 1961.
81.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 65/63. Isolated from a monkey in Malaya on 18th March, 1963.
82.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 425/63. Isolated from a goat in Malaya in 1963.
83.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 41/64. Isolated from a goat in Malaya on 23rd January, 1964.
84.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 105/64. Isolated from a parrot in Malaya on 24th March, 1964.
85.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 86/65. Isolated from a sheep in Malaya on 2nd March, 1965.

Strain No.	Sender, source and further references
86.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 377/65. Isolated from a goat in Malaya on 3rd August, 1965.
87.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 456/65. Isolated from an ox in Malaya on 8th September, 1965.
88.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 535/65. Isolated from a pig in Malaya on 19th October, 1965.
89.	Chief Research Officer, Veterinary Research Institute, Ipoh, Malaya. Ref. 348/66. Isolated from an Orang utan in Malaya on 21st May, 1966.
90.	Mrs. M.A. Reid, Veterinary Laboratory, Department of Agriculture, Port Morsby, Papua, New Guinea. Ref. Papua-1. Probably isolated from cattle in 1959.
91.	Mrs. M.A. Reid, Veterinary Laboratory, Department of Agriculture, Port Morsby, Papua, New Guinea. Probably isolated from cattle in 1959.
92.	Dr. A.D. Alexander, Chief, Department of Veterinary Microbiology, Walter Reed Army Institute for Research, Washington D.C., U.S.A. Ref. 1379. Isolated from the shoulder abscess of a soldier with a history of residence in Panama. Isolated in December, 1957.
93.	Professor P.V. Liu, Department of Microbiology, School of Medicine, University of Louisville, Kentucky, U.S.A. Ref. CDC 1960-2/26/70. Isolated from a human case. See: Am. J. Trop. Med. & Hyg. 1964, 13: 89.

Strain No.	Sender, source and further references
94.	Professor P.V. Liu, Department of Microbiology, School of Medicine, University of Louisville, Kentucky, U.S.A. Ref. CDC 720-2/26/70. Isolated from a human case. See: Am. J. Trop. Med. & Hyg. 1964, 13: 89.
95.	Professor P.V. Liu, Department of Microbiology, School of Medicine, University of Louisville, Kentucky, U.S.A. Ref. CDC 63060-7/12/65. Isolated from a human case. The standard strain at the Communicable Disease Centre, Atlanta, Georgia, U.S.A.
96.	Professor P.V. Liu, Department of Microbiology, School of Medicine, University of Louisville, Kentucky, U.S.A. Ref. 103 (C.Nigg) 7/16/65 Originally received from Dr. Clara Nigg.
97.	Professor P.V. Liu, Department of Microbiology, School of Medicine, University of Louisville, Kentucky, U.S.A. Ref. 118 (C.Nigg) 7/16/65 Originally received from Dr. Clara Nigg.
98.	Professor P.V. Liu, Department of Microbiology, School of Medicine, University of Louisville, Kentucky, U.S.A. Ref. B-1110 - 2/26/20 Originally received from Dr. W.C. Haynes, North Utilisation Research and Development Division, United States Department of Agriculture, Peoria, Illinois, U.S.A.
99.	Professor R.K. MacPherson, School of Public Health and Tropical Medicine, Sydney, New South Wales, Australia. Ref. 30.302 "Hooker". Isolated from a swab taken from an infected elbow of a human, in Australia. Originally received from the Commonwealth Health Laboratory, Darwin in April, 1967.

Strain No.	Sender, source and further references
100.	Professor R.K. MacPherson, School of Public Health and Tropical Medicine, Sydney, New South Wales, Australia. Ref. 30-303 "Angela". Isolated from a leg ulcer of a native child, aged 15 months, in Australia. Originally received from the Commonwealth Health Laboratory, Darwin, in November, 1965.
101.	Professor R.K. MacPherson, School of Public Health and Tropical Medicine, Sydney, New South Wales, Australia. Ref. 30-304 "Harrison". Isolated from the blood culture of a fatal case of a female aged 54 years, in Australia. Originally received from the Commonwealth Health Laboratories, Townsville, Australia, in March, 1968.
102.	Professor R.K. MacPherson, School of Public Health and Tropical Medicine, Sydney, New South Wales, Australia. Ref. 30-305 "Villaflor". Isolated from the liver abscesses of a patient who died after a short illness considered to be "'flu". Originally received from the Commonwealth Health Laboratories, Townsville, Australia in July, 1960.
103.	Professor R.K. MacPherson, School of Public Health and Tropical Medicine, Sydney, New South Wales, Australia. Ref. 30-306 "McQuade". Isolated from the blood culture of a female aged 17 years who died after a 4-week illness. Originally received from the Commonwealth Health Laboratories, Townsville, Australia in April, 1960.
104.	Professor R.K. MacPherson, School of Public Health and Tropical Medicine, Sydney, New South Wales, Australia. Ref. 30-307 "Seatle". Isolated from the pleural effusion of a diabetic male aged 40 years, in Australia. Originally received from the Commonwealth Health Laboratories, Townsville, Australia, in April, 1960.

Strain No.	Sender, source and further references
105.	Professor R.K. MacPherson, School of Public Health and Tropical Medicine, Sydney, New South Wales, Australia. Ref. 30-309 "Lewis". Isolated at post-mortem from the spleen of a male aged 45 years. Lesions were also present in the lungs and liver. Originally received from the Commonwealth Health Laboratories, Townsville, Australia in March, 1960.
106.	Professor R.K. MacPherson, School of Public Health and Tropical Medicine, Sydney, New South Wales, Australia. Ref. 30-310 "Athenasius" isolated from a native child aged 3 years. Originally received from the Commonwealth Health Laboratories, Darwin, Australia in February, 1962.
107.	Professor R.K. MacPherson, School of Public Health and Tropical Medicine, Sydney, New South Wales, Australia. Ref. 30-311 "Douglas". Isolated from the blood culture of a native aged 54 years with gastro-enteritis. Originally received from the Commonwealth Health Laboratories, Cavius, Australia, in February, 1963.
108.	Professor R.K. MacPherson, School of Public Health and Tropical Medicine, Sydney, Australia. Ref. 30-312 "Hitoma". Isolated from urine culture of a human case with a history of acute retention. Originally received from the Commonwealth Health Laboratories, Darwin, Australia, in May, 1963.
109.	Professor R.K. MacPherson, School of Public Health and Tropical Medicine, Sydney, New South Wales, Australia. Ref. 30-313 "Halpin". Isolated from a lung abscess at post-mortem of a man with a history of multiple abscesses for 20 years. Originally received from the Repatriation General Hospital, Concord, Australia in February, 1967.

Strain No.	Sender, source and further references
110.	Professor R.K. MacPherson, School of Public Health and Tropical Medicine, Sydney, New South Wales, Australia. Ref. 30-314 "Lynch". Isolated from the blood culture of a man in Australia. Originally received from the Commonwealth Health Laboratories, Darwin, Australia in March, 1967.
111.	Animal Disease Research Centre, Sabah, Malaysia. Rev. VL 296/70 isolated from the lung abscess of a goat on 6th July, 1970.
112.	Animal Disease Research Centre, Sabah, Malaysia. Ref. VL 300/70/A. Isolated from the lung abscess of a pig on 14th July, 1970.
113.	Animal Disease Research Centre, Sabah, Malaysia. Ref. VL 343/70. Isolated from lymph nodes of a pig in Papar, Sabah, on 2nd October, 1970.
114.	Animal Disease Research Centre, Sabah, Malaysia. Ref. VL 350/70. Isolated from the spleen of a pig in Papar, Sabah on 15th October, 1970.
115.	Animal Disease Research Centre, Sabah, Malaysia. Ref. VL 348/70. Isolated from the kidney of a pig foetus in Papar, Sabah on 21st October, 1970.
116.	Animal Disease Research Centre, Sabah, Malaysia. Ref. VL 421/70. Isolated from a lung abscess of a pig in Tenom, Sabah, on 20th December, 1970.
117.	Animal Disease Research Centre, Sabah, Malaysia. Ref. VL 53/71. Isolated from the spleen of a pig on 9th February, 1971.
118.	Dr. Jan Nari, Animal Research Institute, Bogor, Indonesia. A laboratory stock culture. Source and date of isolation not stated.

APPENDIX A.2: Strains of Ps.aeruginosa used in the study

Strain	Source
Ps.aeruginosa (NCTC 7244)	Department of Veterinary Pathology, University of Edinburgh.
Ps.aeruginosa (5781)	Dr. T.V. Alper, M.R.C. Experimental Radiopathology Unit, Hammersmith Hospital, London, W.12. Ref. J.Bact., 1972, 110: 823-830)
Ps.aeruginosa (IC)	Dr. T.V. Alper, M.R.C. Experimental Radiopathology Unit, Hammersmith Hospital, London, W.12. (Ref. J.Bact., 1972, 110: 823-830)
Ps.aeruginosa (HCR-5)	Dr. T.V. Alper, M.R.C. Experimental Radiopathology Unit, Hammersmith Hospital, London, W.12. (Ref. J.Bact., 1972, 110: 823-830)
Ps.aeruginosa (HCR-13)	Dr. T.V. Alper, M.R.C. Experimental Radiopathology Unit, Hammersmith Hospital, London, W.12. (Ref. J.Bact., 1972, 110: 823-830)
Ps.aeruginosa (P.10)	Dr. J. Fournier, Pasteur Institute, Paris.

APPENDIX A.3: Strains of other bacteria used in the study

Strain	Source
Escherichia coli (NCTC 9001)	National Collection of Type Cultures, United Kingdom.
Salmonella typhimurium (NCTC 74)	National Collection of Type Cultures, United Kingdom.
Salmonella typhi	The Royal (Dick) School of Veterinary Studies, Edinburgh.
Shigella sonnei (NCTC 8220)	National Collection of Type Cultures, United Kingdom.
Shigella dysenteriae	The Royal (Dick) School of Veterinary Studies, Edinburgh.
Klebsiella aerogenes (NCTC 418)	National Collection of Type Cultures, United Kingdom.
Klebsiella pneumoniae	The Royal (Dick) School of Veterinary Studies, Edinburgh.
Alkaligenes faecalis (NCTC 655)	National Collection of Type Cultures, United Kingdom.
Enterobacter cloacae (NCTC 10005)	National Collection of Type Cultures, United Kingdom.
Achromobacter anitratus (NCTC 7844)	National Collection of Type Cultures, United Kingdom.
Aeromonas hydrophila (NCTC 7810)	National Collection of Type Cultures, United Kingdom.
Bacillus subtilis (NCTC 3610)	National Collection of Type Cultures, United Kingdom.

Strain	Source
Staphylococcus aureus (NCTC 8532)	National Collection of Type Cultures, United Kingdom.
Streptococcus faecalis (NCTC 8213)	National Collection of Type Cultures, United Kingdom.
Streptococcus zooepidemicus	The Royal (Dick) School of Veterinary Studies, Edinburgh.
Corynebacterium pyogenes	The Royal (Dick) School of Veterinary Studies, Edinburgh.
Haemophilus influenzae	The Royal (Dick) School of Veterinary Studies, Edinburgh.
Pasteurella septica (1476/60)	The Royal (Dick) School of Veterinary Studies, Edinburgh.
Pasteurella haemolytica	The Royal (Dick) School of Veterinary Studies, Edinburgh.
Serratia marcescens	Department of Bacteriology, Medical School, Edinburgh.
Sarcina lutea	Department of Bacteriology, Medical School, Edinburgh.
Chromobacterium violaceum (LD 509/70)	Animal Disease Research Centre, Sabah, Malaysia. Isolated from the heart blood of a gibbon.

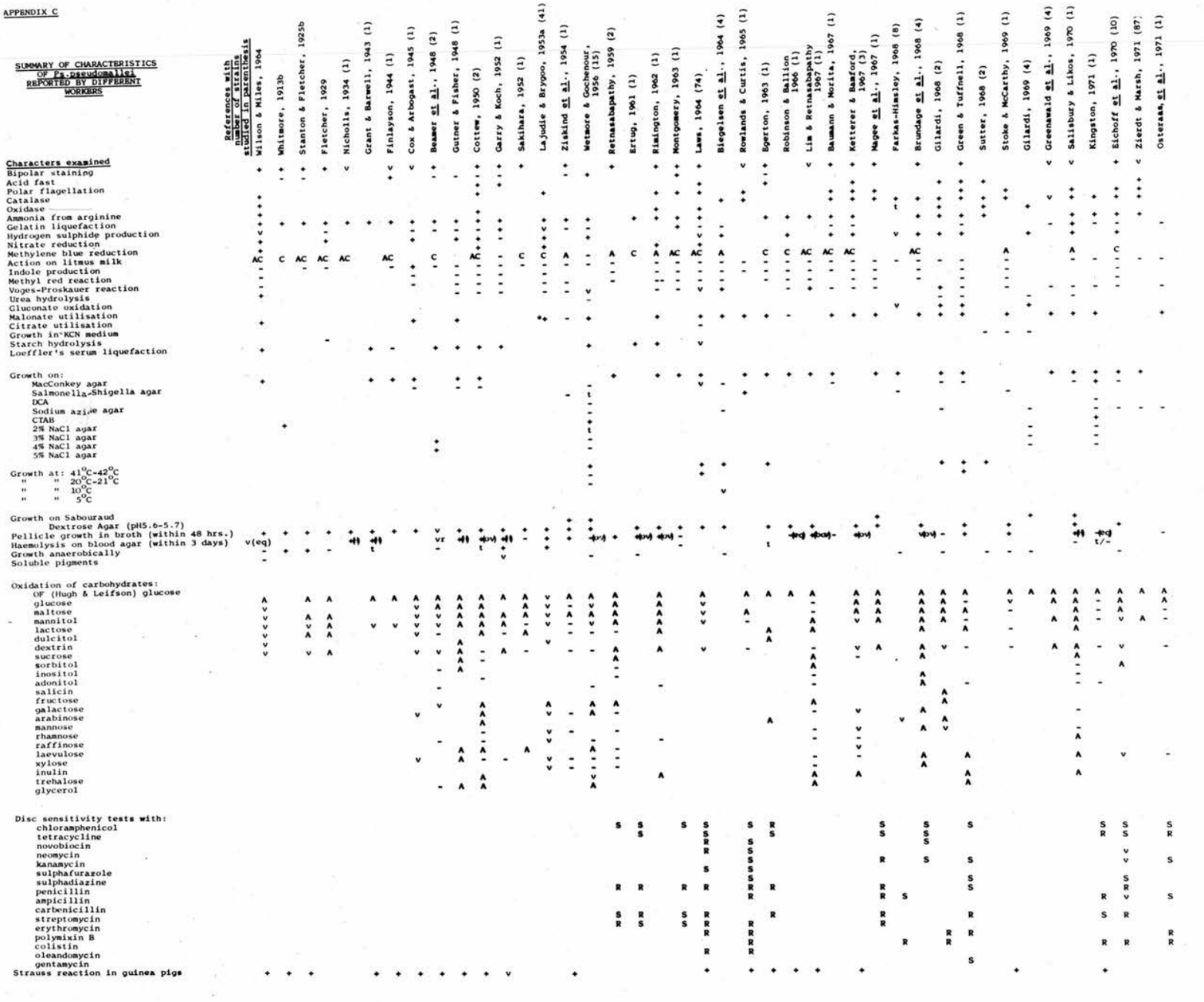
APPENDIX B: Particulars of 24 strains of Ps.pseudomallei tested for sensitivity to antimicrobial agents by the tube dilution test (MIC)

Strain	Source	
1	Sabah:	Isolated from the lung of a pig in 1969 (VL411/69)
2	Sabah:	Isolated from the lung of a sheep in 1969 (VL459/69)
3	Sabah:	Isolated from the lung of a pig in 1969 (VL463/69)
4	Sabah:	Isolated from the lung of a goat in 1969 (VL524/69)
5	Sabah:	Isolated from the spleen of a goat in 1969 (VL498/69)
15	Ecuador:	Isolated from man. Received from Dr. Fournier ("4889")
29	Sabah:	Isolated from the spleen of a rabbit in 1969 (VL622/69)
30	Sabah:	Isolated from the spleen of an orang-utan in 1969 (VL646/69)
32-R	NCTC:	Isolated from a rat by Fletcher in 1923 (NCTC 1688)
34	NCTC:	Isolated from a laboratory monkey (NCTC 4846)
35	NCTC:	Isolated from an abscess in a human (NCTC 7600)
36	NCTC:	Isolated from a West African soldier who served in Burma (NCTC 7383)
37	NCTC:	Isolated from a patient at Guy's Hospital, U.K. in 1948 (NCTC 7431)
38	NCTC:	Isolated from a sheep (NCTC 8016)

Strain	Source	
39	NCTC:	Isolated from a patient at SEAC Laboratory in Singapore in 1946 (NCTC 8707)
41	NCTC:	Isolated from a soldier at Kinrara Military Hospital (NCTC 10274)
42	NCTC:	Isolated from a horse in Malaya (NCTC 10276)
111	Sabah:	Isolated from the lung of a goat in 1970 (VL296/70)
112	Sabah:	Isolated from the lung of a pig in 1970 (VL300/70)
113	Sabah:	Isolated from the lymph node of a pig in 1970 (VL343/70)
114	Sabah:	Isolated from the lymph node of a pig in 1970 (VL350/70)
115	Sabah:	Isolated from the kidney of a pig foetus in 1970 (VL348/50)
116	Sabah:	Isolated from the lung of a pig in 1970 (VL421/70)
117	Sabah:	Isolated from the spleen of a pig in 1970 (VL153/71)

APPENDIX C

SUMMARY OF CHARACTERISTICS OF Ps.pseudomallei REPORTED BY DIFFERENT WORKERS



Abreviations:-

A = acid
C = clot
AC = acid and clot
t = partial growth
v = variable
S = sensitive
R = resistant

eq = horse blood agar
ov = sheep blood agar
bov = ox blood agar
r = rabbit
H = human
* = Two of 41 strains did not utilise citrate

\+ = positive reaction
\- = negative reaction

Note: 1. The data are from selected references to show the methods that have been used in various laboratories to identify Ps.pseudomallei.

2. Different workers have used different tests and different reading intervals. These details have not been indicated.

3. The results of an extensive range of tests tried out by Redfearn, Palleroni and Stanier (1966) have not been included.

APPENDIX D: Recovery of phage from 27 lysogenic strains of Pseudomonas pseudomallei tested against 10 "indicator" strains of Ps.pseudomallei randomly chosen

Lysogenic strain	"Indicator" strain										Phage isolated and propagated on	Maximum dilution	
	2	4	13	15	17	27	33	74	79	83		confluent lysis	plaque forming units
3-SR	+	+		+			+			+	15-M	undiluted	n.r.
7-S		+			+						17-S	undiluted	n.r.
11-SR*					+						17-S	undiluted	n.r.
14-S	+						+			+	83-S	undiluted	n.r.
18-M	+	+									2-SR	10^{-5}	10^{-8}
19-R*	+	+				+					27-SR	undiluted	n.r.
22-S	+					+					2-SR	undiluted	n.r.
23-S	+	+			+						2-SR	undiluted	n.r.
28-S	+	+		+		+			+		4-SR	10^{-5}	10^{-7}
32-R	+	+		+							15-M	undiluted	n.r.
37-S		+	+		+		+				33-SR	10^{-2}	10^{-5}
38-S		+	+	+	+			+			13-S	undiluted	n.r.
39-M		+									4-SR	undiluted	n.r.
44-S*		+				+		+			74-S	undiluted	n.r.
46-S	+	+		+	+		+				2-SR	10^{-4}	10^{-7}
48-M*	+	+		+	+		+				17-S	undiluted	n.r.
49-M	+	+			+	+	+	+	+	+	2-SR	10^{-4}	10^{-7}
50-SR		+		+	+		+				4-SR	10^{-3}	10^{-6}
71-M	+	+				+	+				33-SR	10^{-2}	10^{-5}
75-M	+	+		+		+	+				2-SR	10^{-4}	10^{-7}
80-M		+			+		+				33-SR	undiluted	n.r.
85-R		+			+						17-S	undiluted	n.r.
104-S		+		+			+				4-SR	10^{-3}	10^{-6}
108-SR	+					+					2-SR	10^{-3}	10^{-6}
109-SR										+	83-S	10^{-1}	10^{-5}
116-SR	+	+					+				33-SR	10^{-4}	10^{-7}
117-SR	+	+									4-SR	10^{-3}	10^{-6}

Explanatory: + = phage activity * = UV induced culture n.r. = not recorded

PLATE 1

Plate 1. Gram-stained preparation of an 'SR' type culture showing the typical bacilli with some cells showing bipolar staining affinities.

PLATE 2

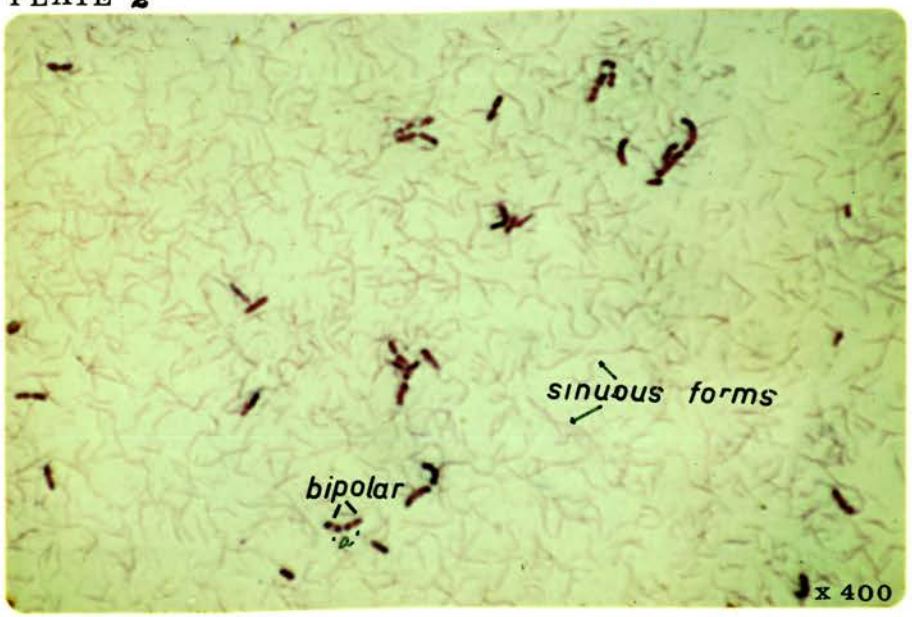


Plate 2. Abundant, faintly stained sinuous forms in a Giemsa stained preparation from an 'SR' culture grown on 2% sodium chloride agar. Note also the darkly-stained bipolar cells ('a') and the curved rods.

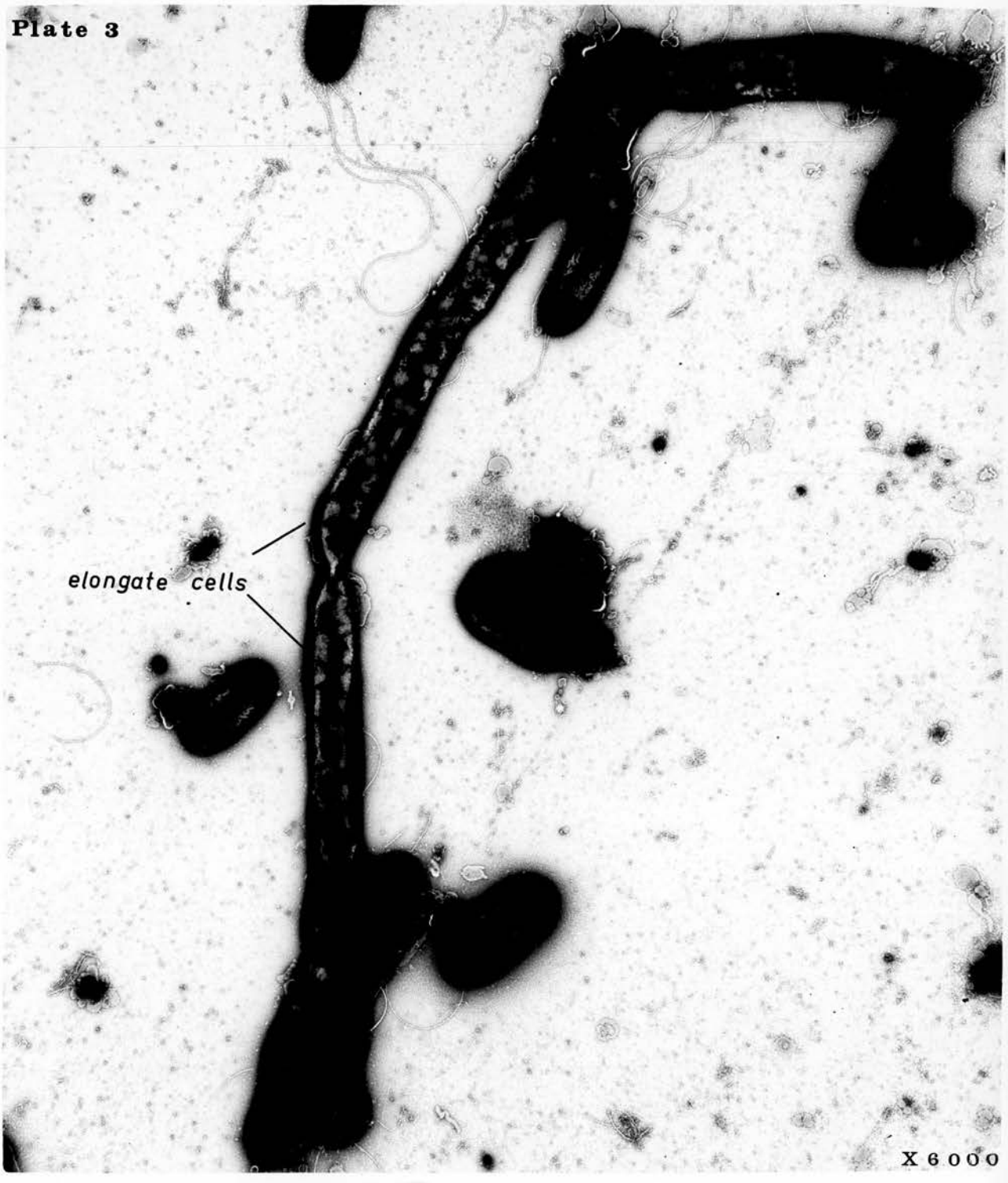


Plate 3. The elongate cells seen in a culture of Ps.pseudomallei grown on sodium chloride agar.

Plate 4a

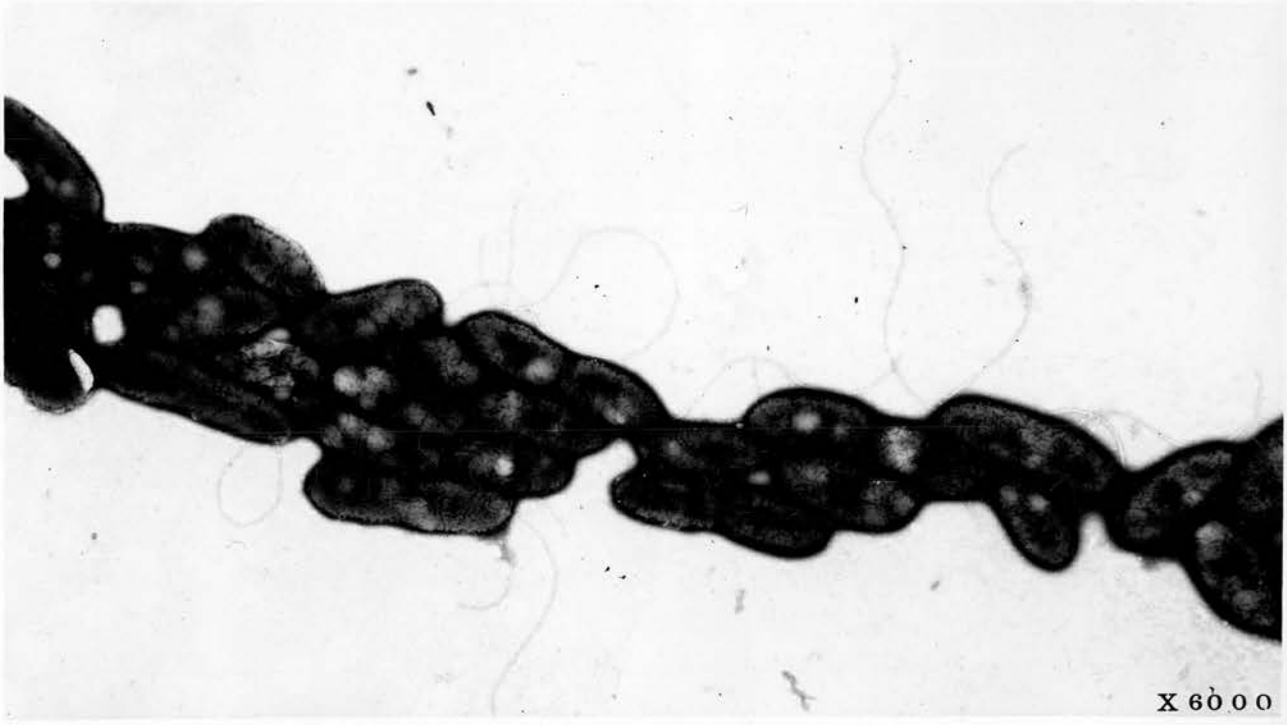


Plate 4b

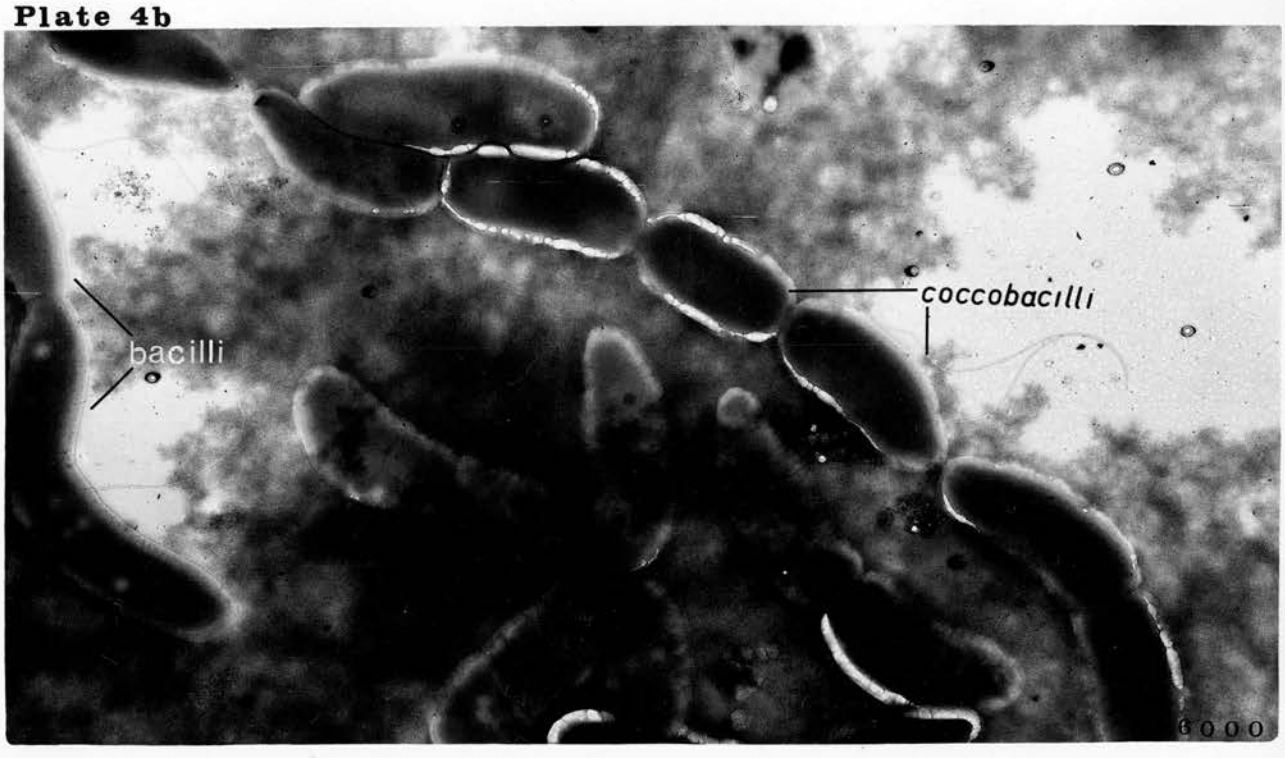


Plate 4a. Chains of coccobacilli.
(Top)

Plate 4b. Chains of coccobacilli and
(Bottom) bacilli. E.M.

Plate 5

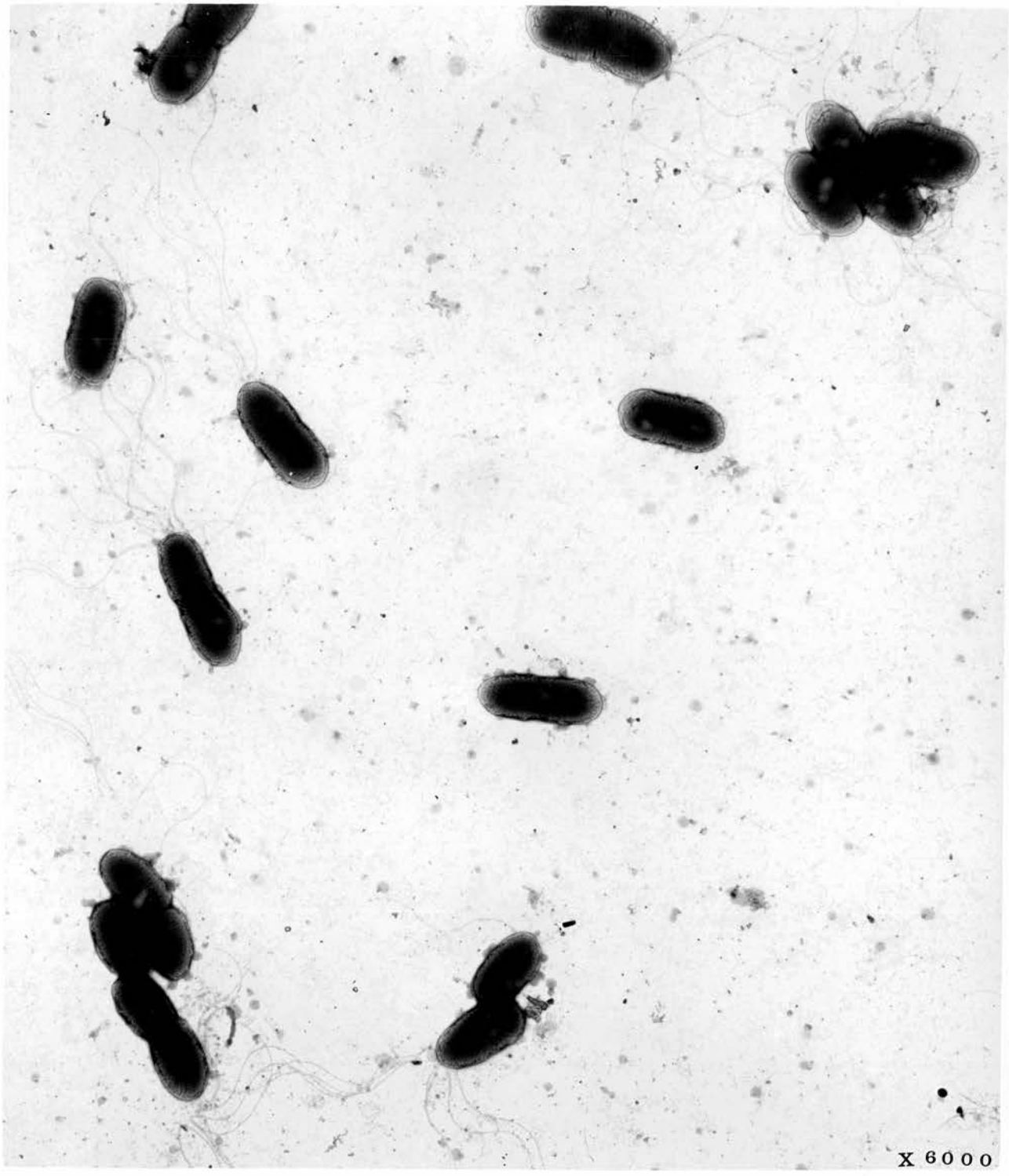


Plate 5. Cells showing multitrichous flagella at one pole and some atrichous cells and cells with a single flagellum (E.M.)

Plate 6

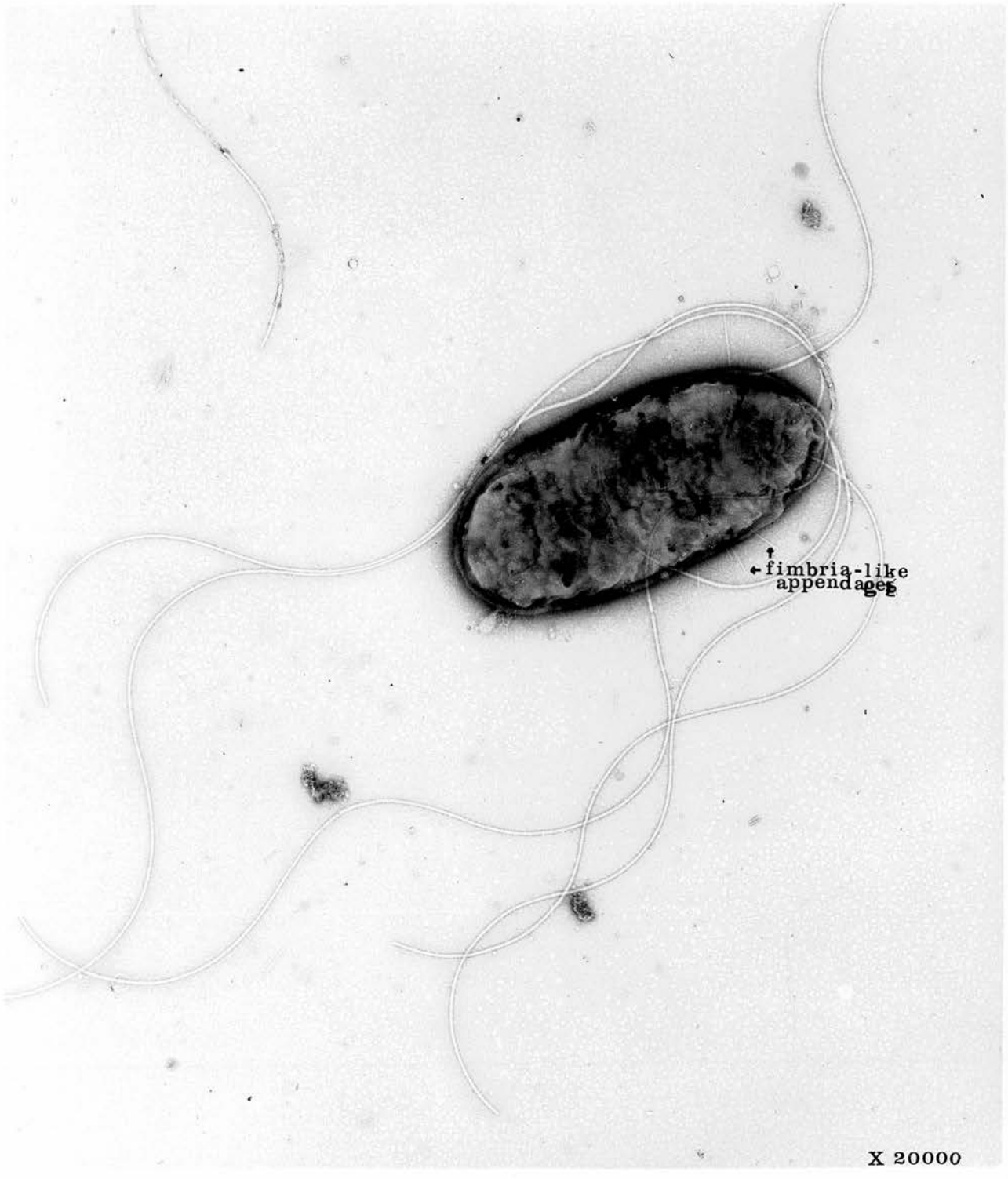


Plate 6. A polar flagellated cell showing fimbriae-like appendages. See tiny strands arising peritrichously. (E.M.)

Plate 7

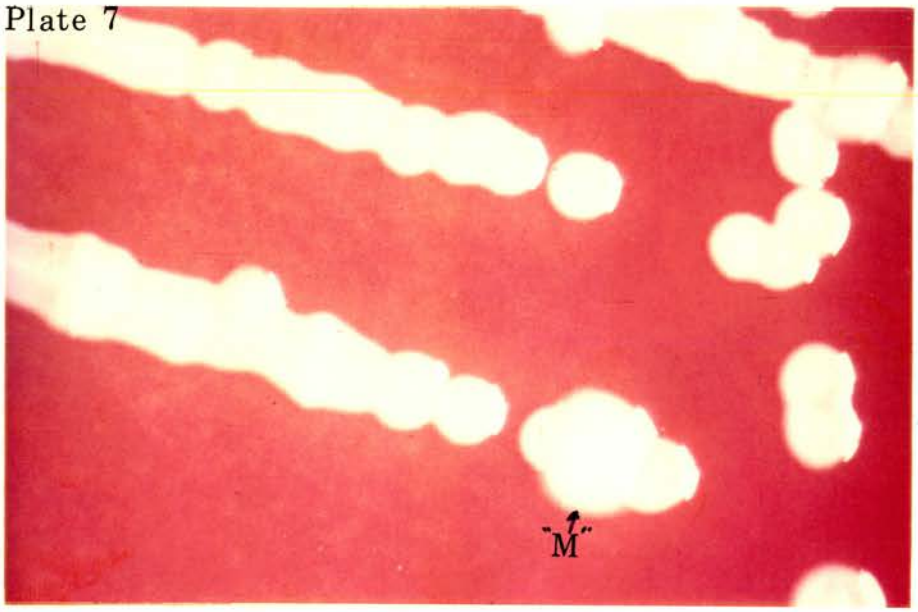


Plate 7. "M" type among smaller "S" colonies. (blood agar)

Plate 8

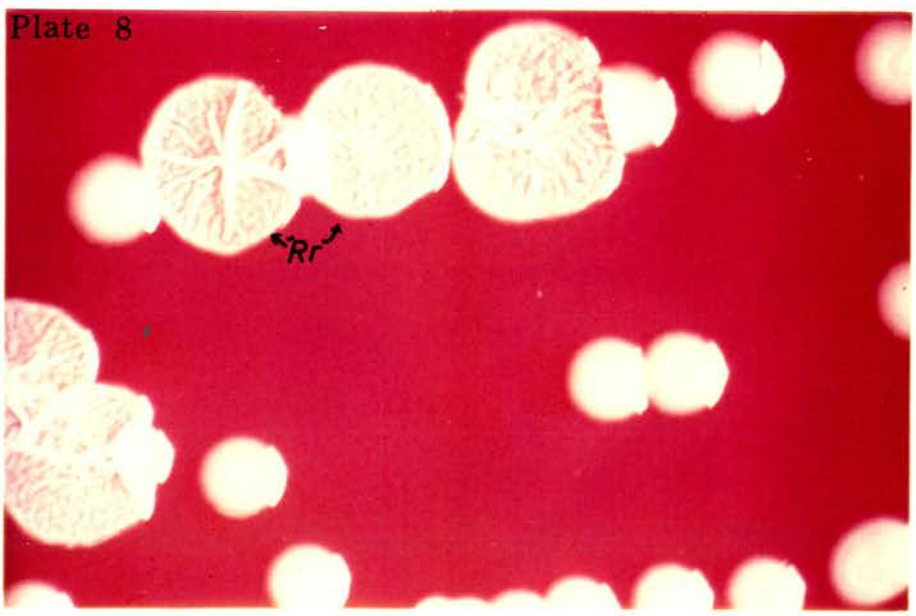


Plate 8. "Rr" type colonies among "S" colonies. (blood agar)

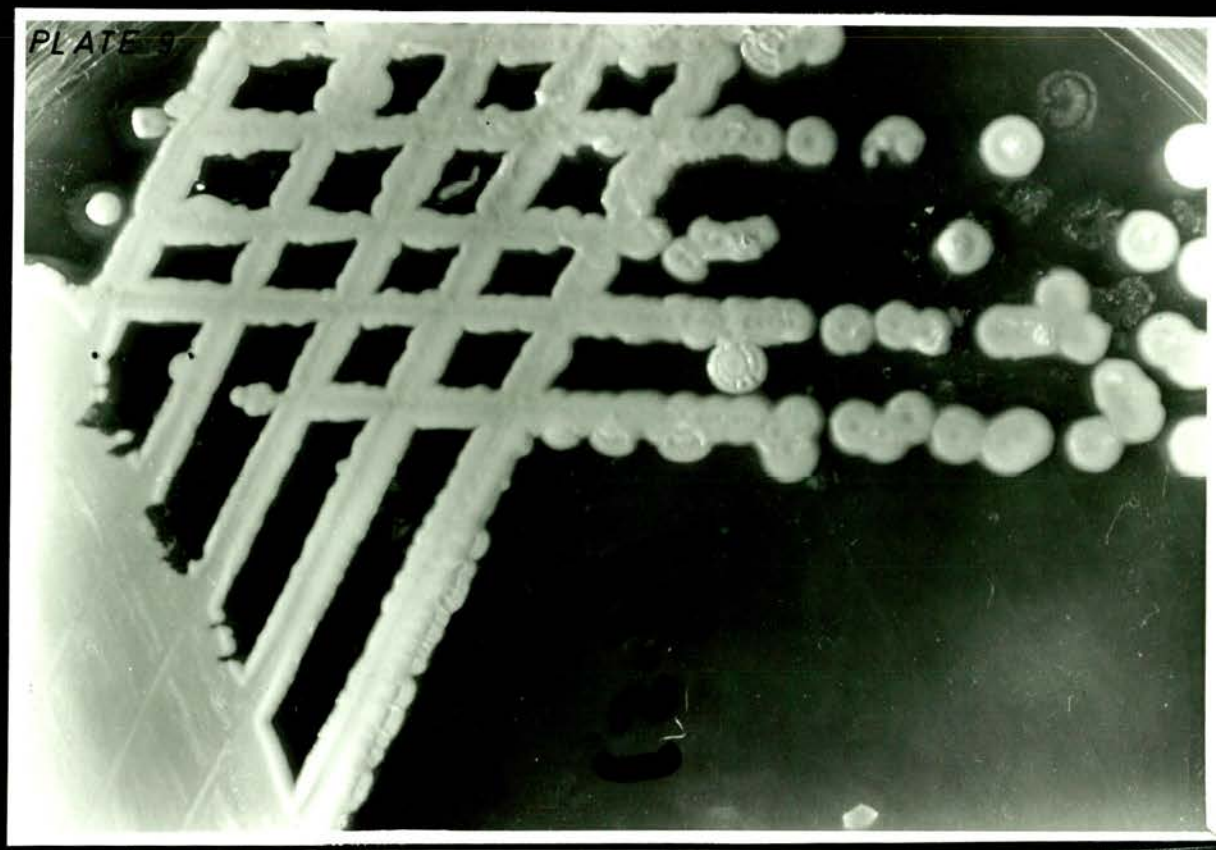


Plate 9. "SR" type colonies at 24 hours. (nutrient agar. x3)

Plate 10

Plate 10. A colony of the "SR" type when incubated for 48-72 hours.

Plate 11a

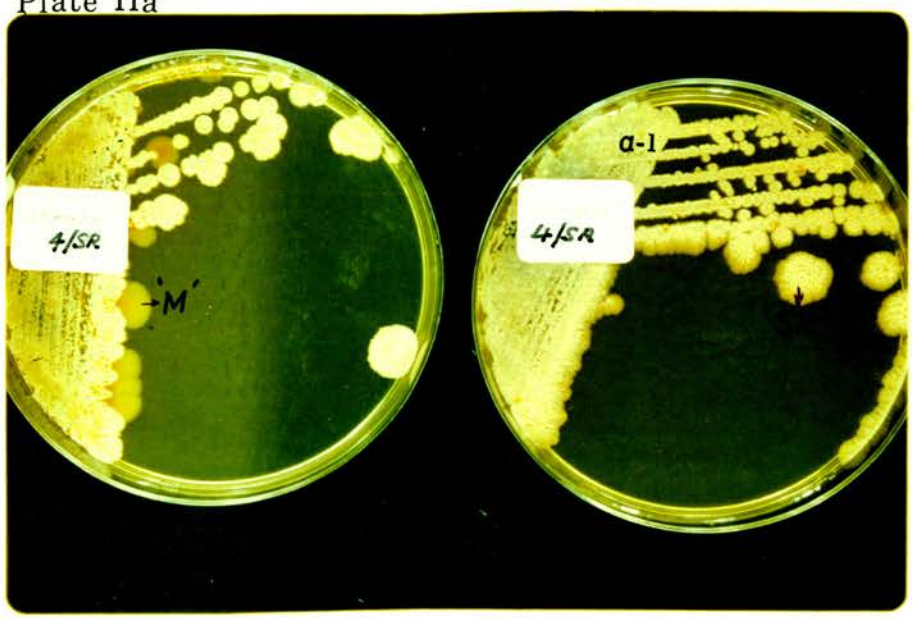


Plate 11a. (1) "SR" colonies of Ps.pseudomallei strain 4-SR at 96 hours.
(2) "M" variants emerging from Ps.pseudomallei strain 4-SR. Note the tan colour of the "M" variant.
(nutrient agar X $\frac{2}{3}$)

PLATE 11b

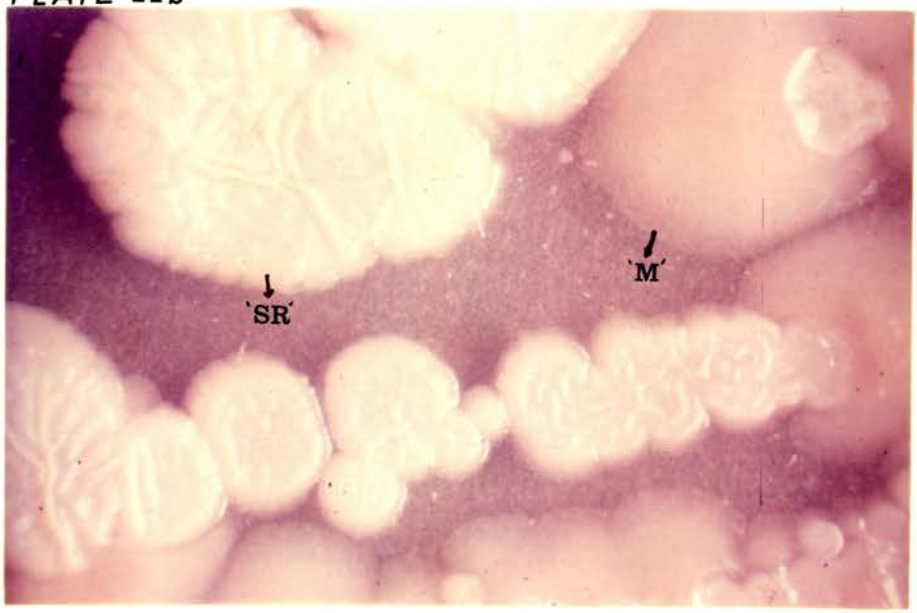


Plate 11b "M" variant from Ps.pseudomallei strain 2.

Plate 12

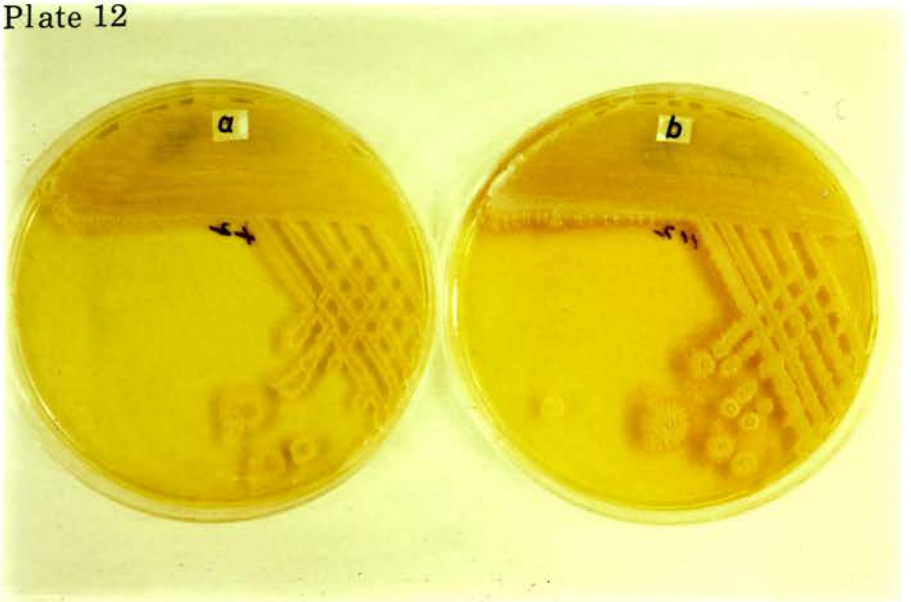


Plate 12. (1) Ps.pseudomallei strain 115-SR grown for 96 hours.
(2) Ps.pseudomallei strain 45-SR grown for 96 hours.
Note the colony colorations.
(nutrient agar x0·6)

Plate 13. 'R' type at 24 hours' incubation.
(nutrient agar. × 1·1)

Plate 14. 'R' type at 72 hours' incubation.
(nutrient agar. X3)

PLATE 15

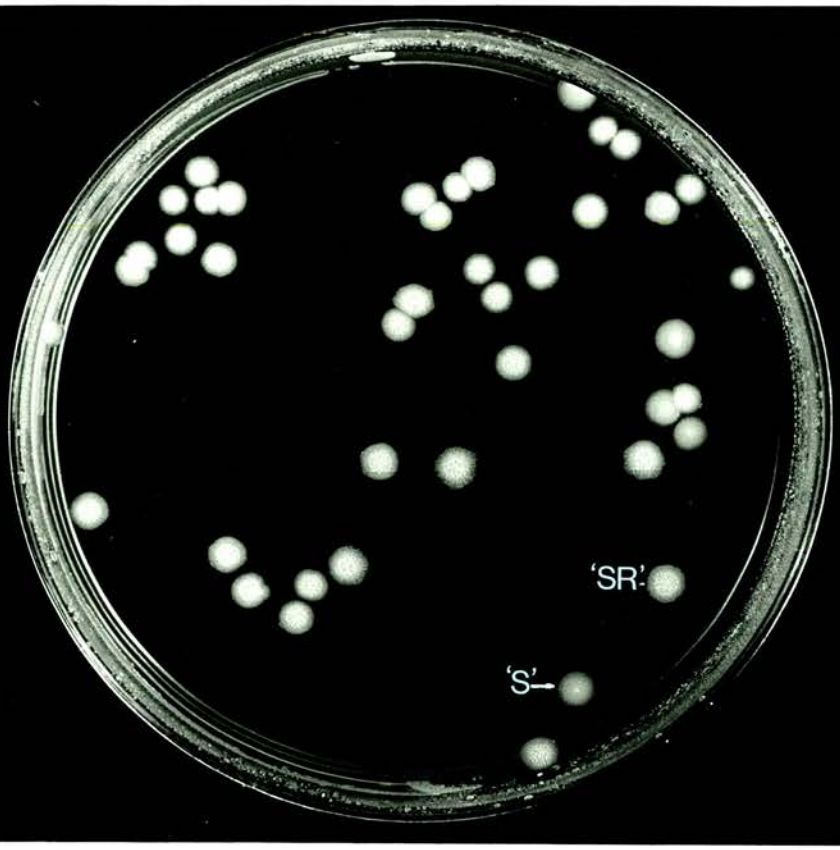


Plate 15. "S" colonies that have risen in a culture of Ps.pseudomallei strain 4-SR. (nutrient agar x 1·2)

PLATE 16a

Plate 16a. (1) The scanty growth observed in a culture of Ps pseudomallei strain 15-M. cf. 25/S on R. (nutrient agar. x 0·7)

PLATE 16b

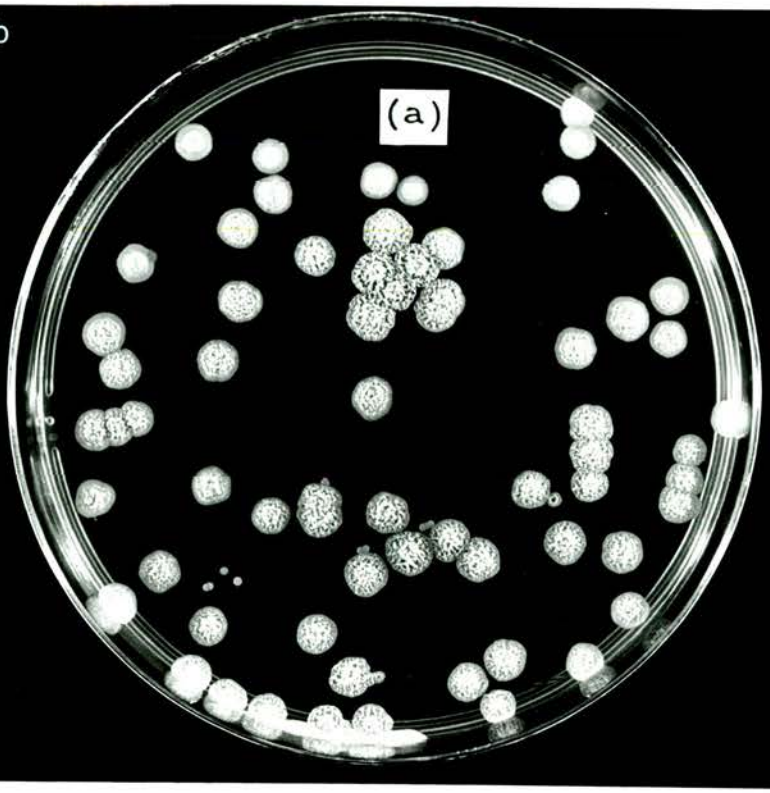


Plate 16b. Dissociant colonies from an "SR" type culture. (nutrient agar x 1·2)

PLATE 16c

Plate 16c. Dissociant colonies from an "S" type culture. (nutrient agar x 1·2)

Plate 16d. Secondary dissociant 4"Rr" redissociating to "S". (nutrient agar. X 1·2)

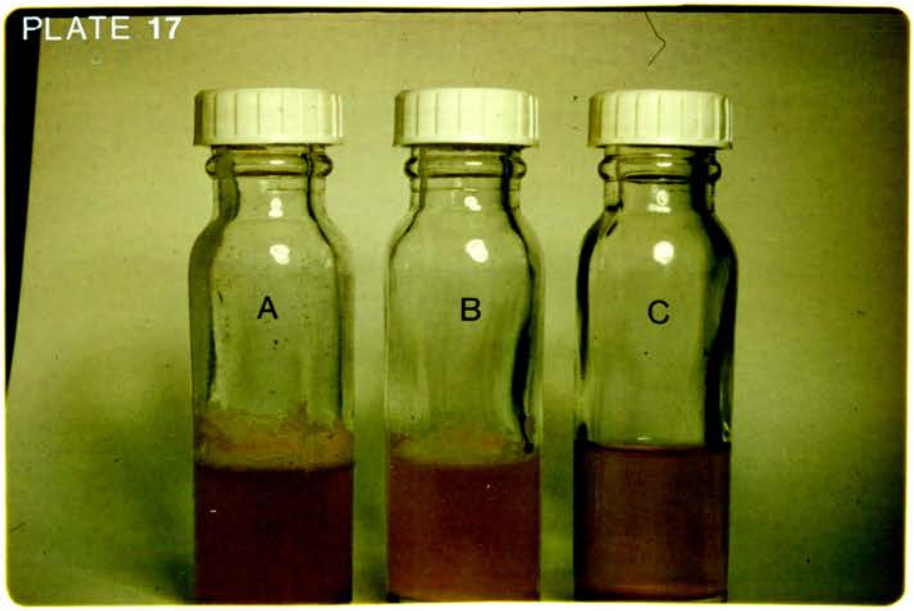


Plate 17. A & B: Pellicle formation in "SR" type cultures of Ps.pseudomallei grown in nutrient broth for 48-72 hours

C: Absence of pellicle growth in an "S" type culture of Ps.pseudomallei grown for 48 hours.

PLATE 18

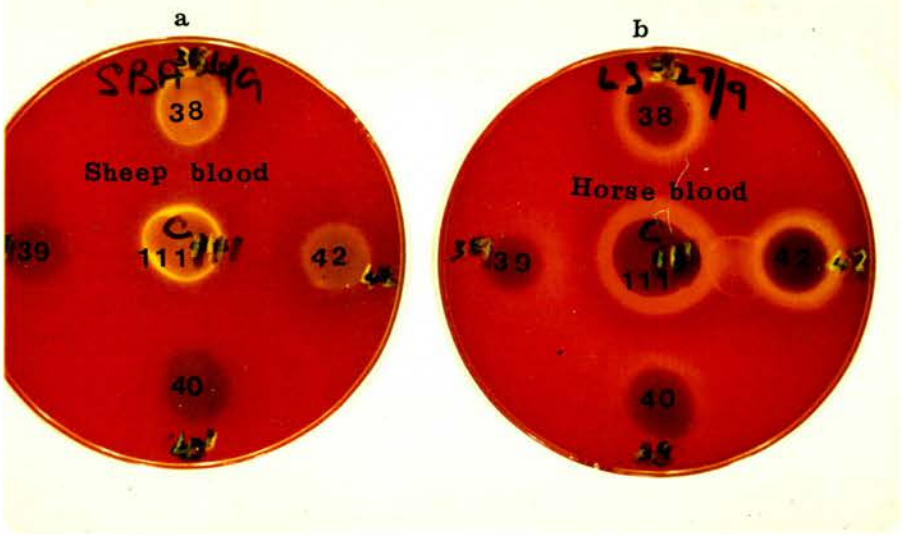


Plate 18a,b. Haemolytic activity of 5 strains of Ps.pseudomallei (strains 38; 39; 40; 42 and 111) grown on sheep blood and on horse blood agar for 3 days at 37°C.

PLATE 19

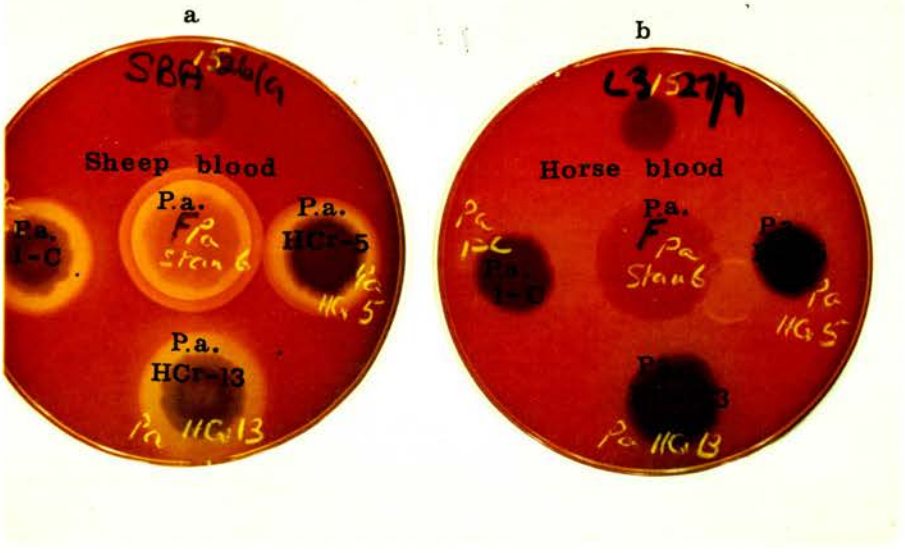


Plate 19a,b. The haemolytic activity of 4 strains of Ps.aeruginosa (HCr-5, HCr-13, 1-C & P.a.5781) and one strain of Ps.pseudomallei (strain 15) on horse blood agar and on sheep blood agar.

Plate 20a

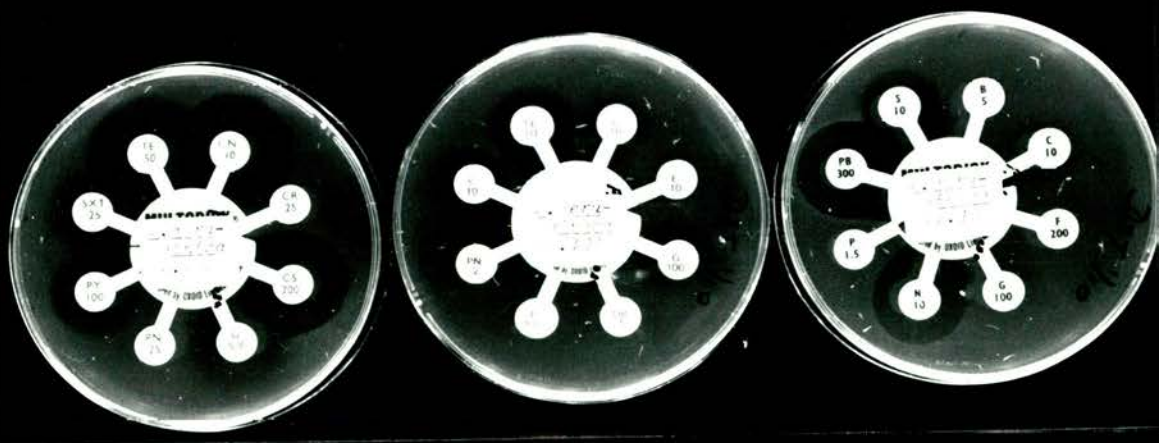


Plate 20b

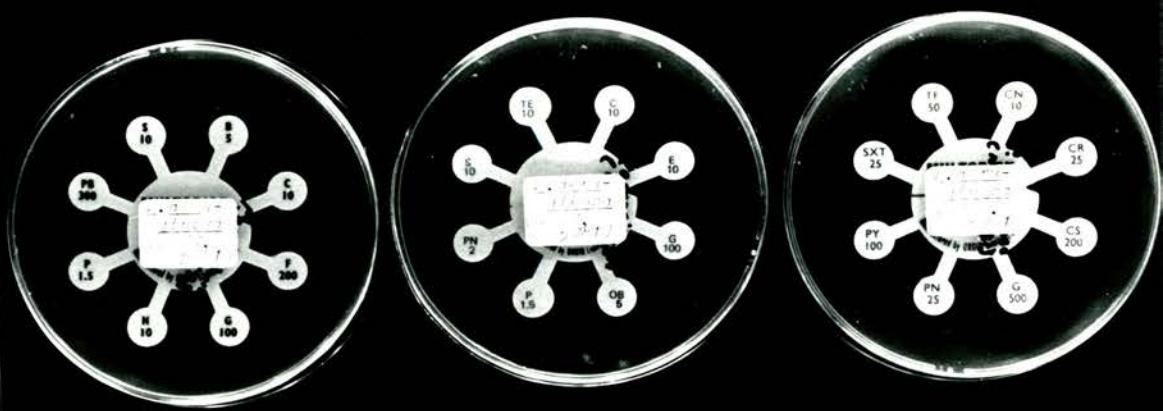
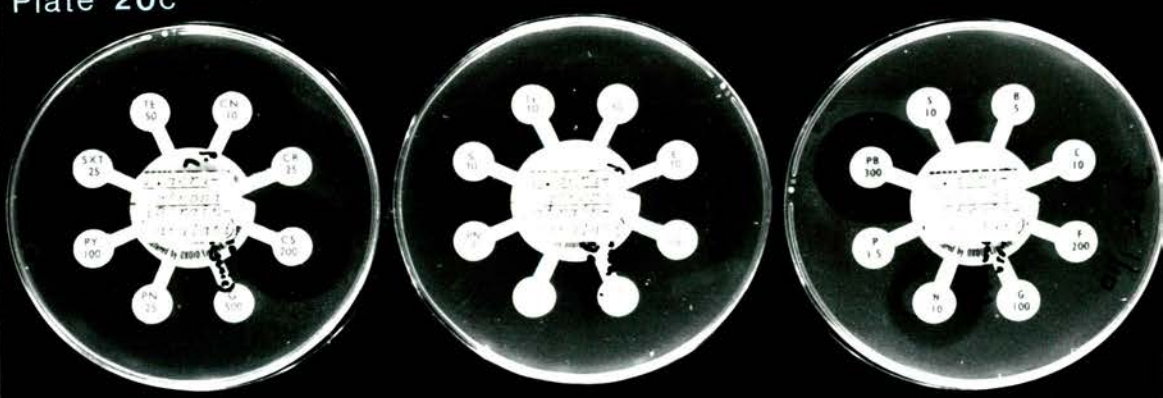


Plate 20c



Plates 20a,b,c. Sensitivity patterns of 3 strains of Ps.aeruginosa against Oxoid "Multodisks". Te = Tetracycline; CN = Gentamycin; CR = Cephaloridine; S = Streptomycin; CS = Carbenicillin; SXT = Sulphamethoxazole/ Trimethoprim; E = Erythromycin; OB = Cloxacillin; B = Bacitracin; F = Nitrofurantoin; N = Neomycin; PB = Polymixin B.

PLATE 21a

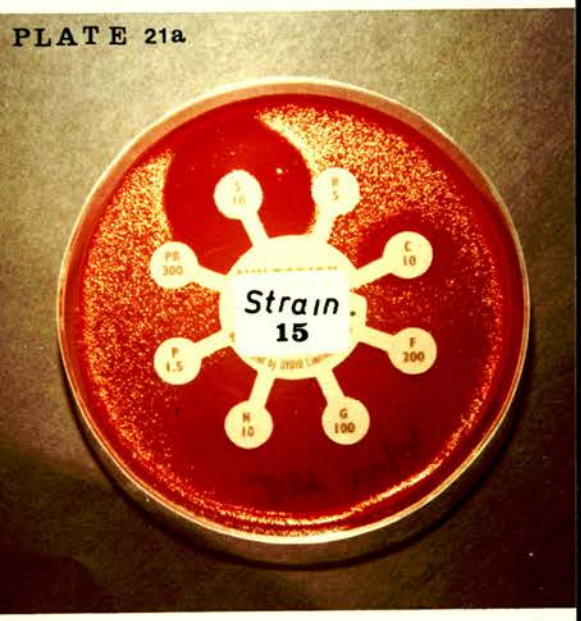


PLATE 21b

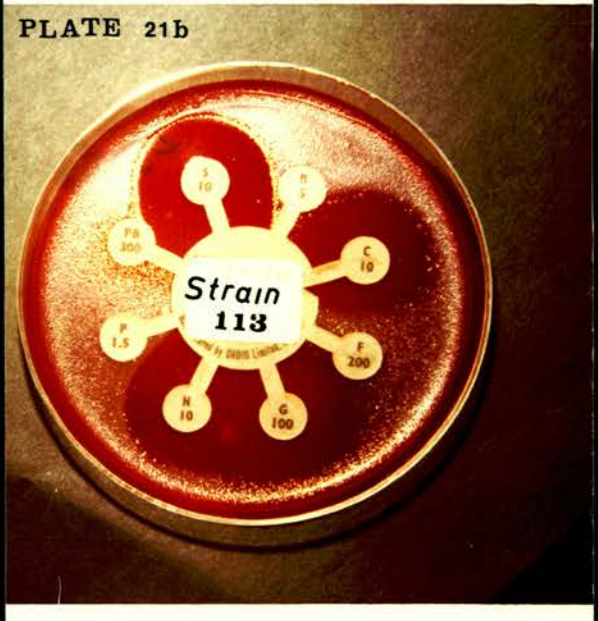
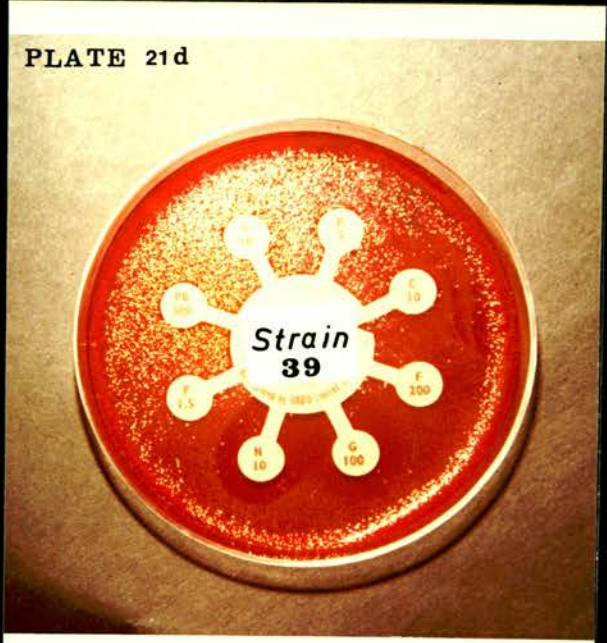


PLATE 21c

PLATE 21d



Plates 21a,b,c & d. Sensitivity patterns of 4 strains of Ps.pseudomallei (strains 15, 113, 41 and 39) against Oxoid "Multodisks". Note the sensitivity of strains 15 and 113 to streptomycin.
Antibiotic discs: S = Streptomycin;
B = Bacitracin; C = Chloramphenicol;
F = Furazolidone; G = Sulphafurazole;
N = Neomycin; P = Penicillin; PB = Polymixin B.

PLATE 22a

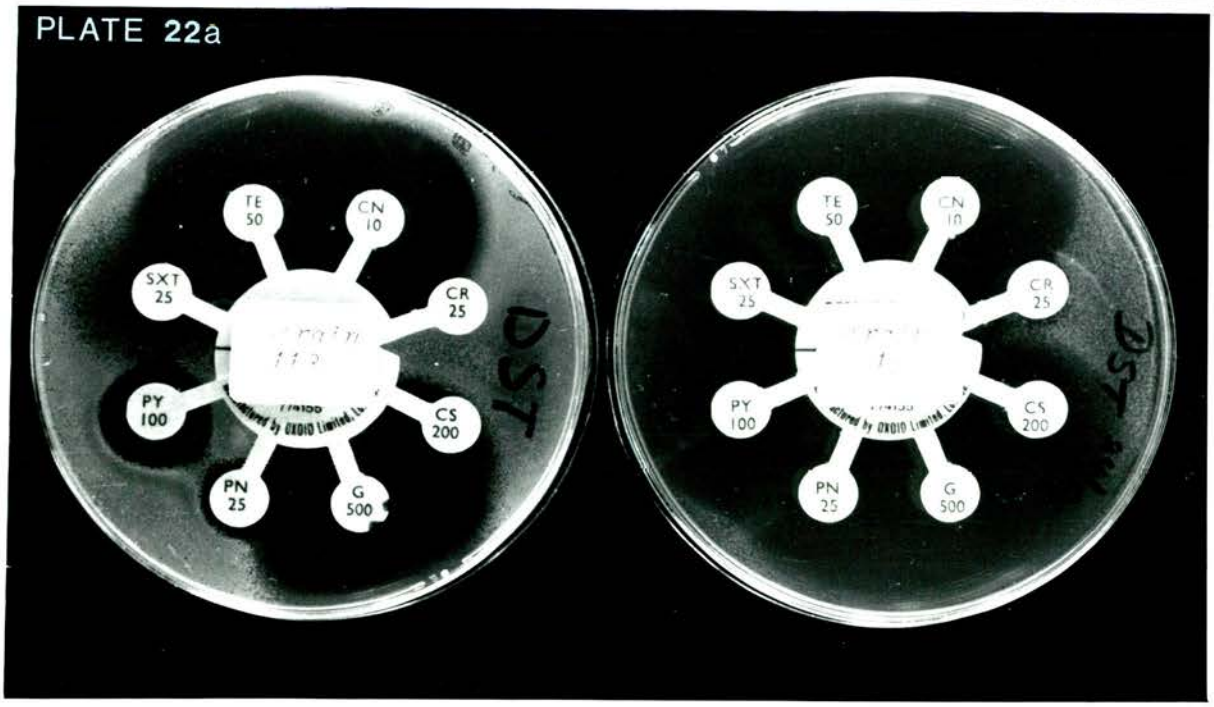
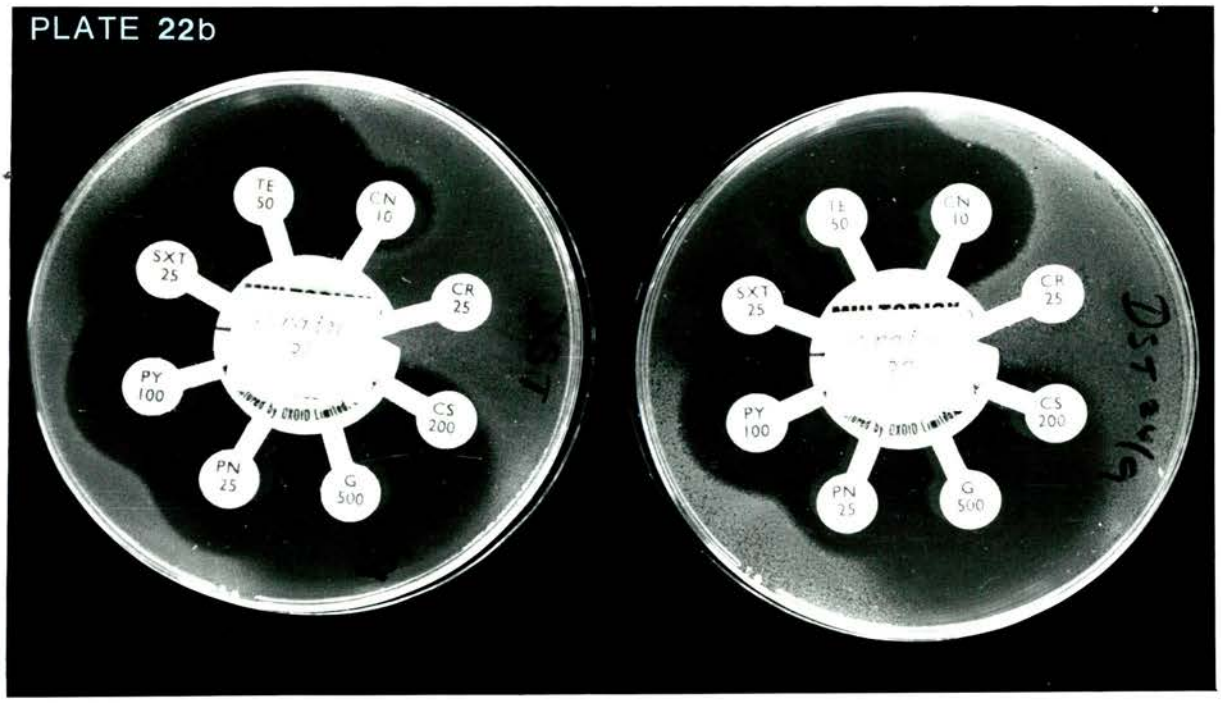


PLATE 22b



Plates 22a & 22b. Sensitivity patterns of 4 strains of Ps.pseudomallei (strains 113, 15, 38 & 32) against Oxoid "Multodisks". Note the relatively greater sensitivity of strains 113 and 15 to gentamycin and the sensitivity of strain 15 to colistin.
Antibiotic discs: TE = Tetracycline; CN = Gentamycin; CR = Cephaloridine; CS = Colistin methane sulphonate; G = Sulphafurazole; PN = Ampicillin; PY = Carbenicillir SXT = Sulphamethoxazole/Trimethoprim.

PLATE 23a

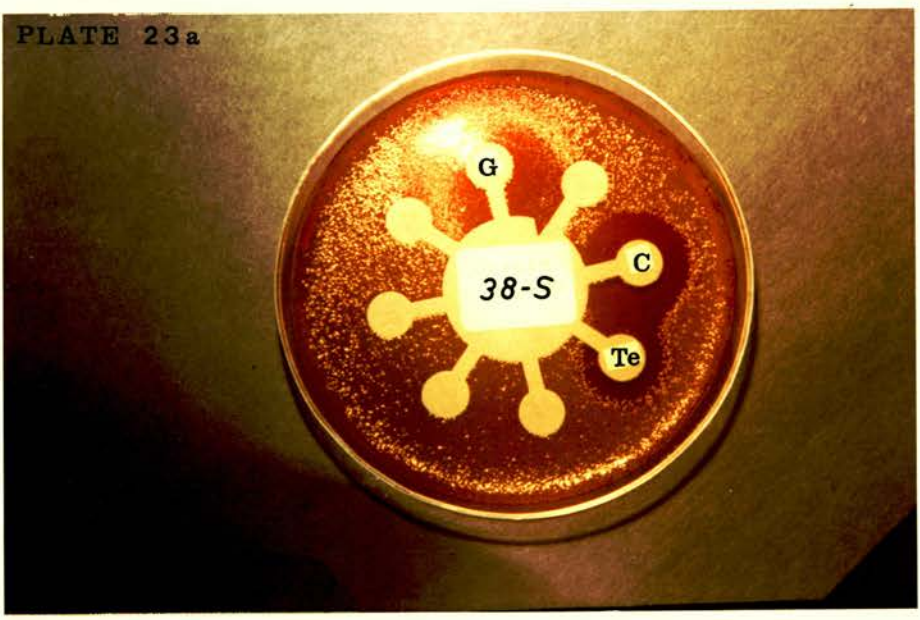
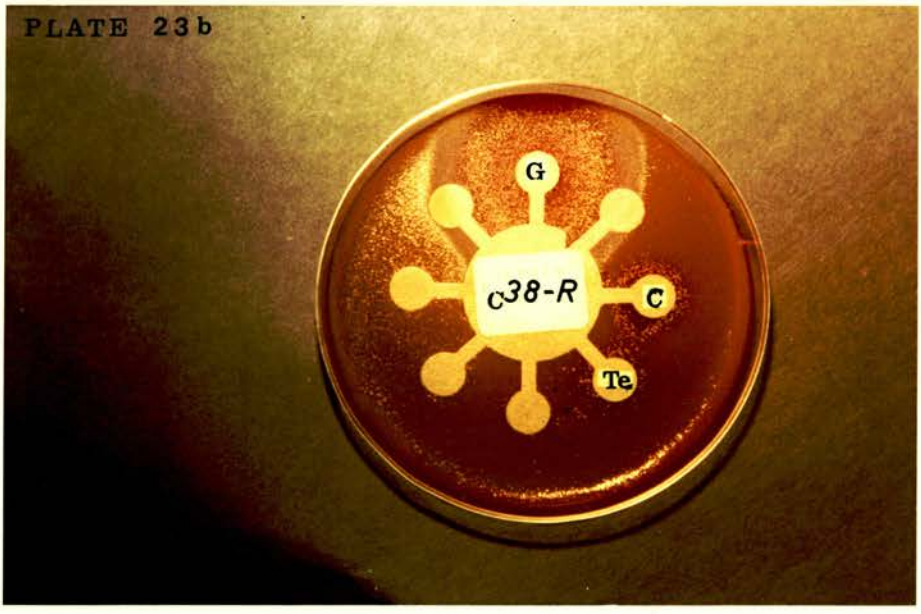


PLATE 23b



Plates 23a & b. The sensitivity of 2 dissociants from the same strain (Ps.pseudomallei strain 38)to an identical "Multodisk".
Antibiotic discs: G = Sulphafurazole; C = Chloramphenicol; TE = Tetracycline.

PLATE 24

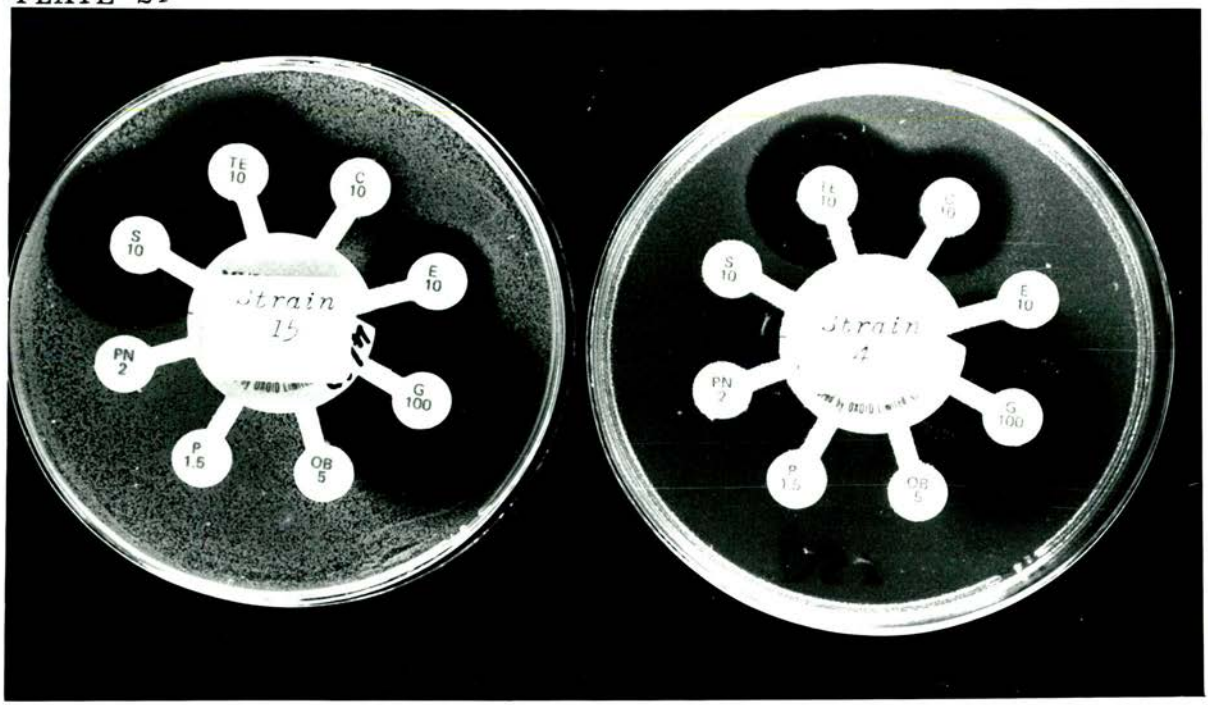


Plate 24a,b. Two strains of Ps.pseudomallei (Strain 4-Sabah and strain 15-Ecuador).

Strain 4 represents the typical sensitivity pattern of Ps.pseudomallei tested against Oxoid Multodisks.

Note the sensitivity of Strain 15 to streptomycin and to erythromycin and its low sensitivity to chloramphenicol.

Antibiotic discs: Te = Tetracycline; C = Chloramphenicol; E = Erythromycin; G = Sulphafurazole; OB = Cloxacillin; P = Penicillin; PN = Ampicillin

PLATE 25a

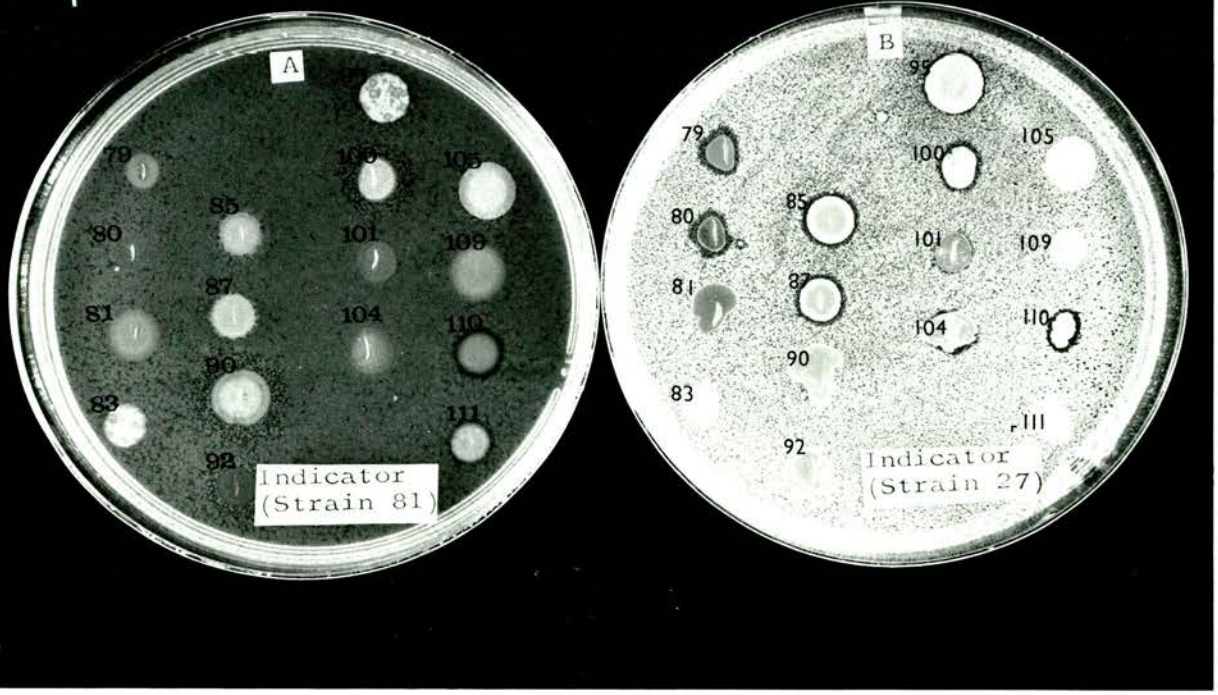
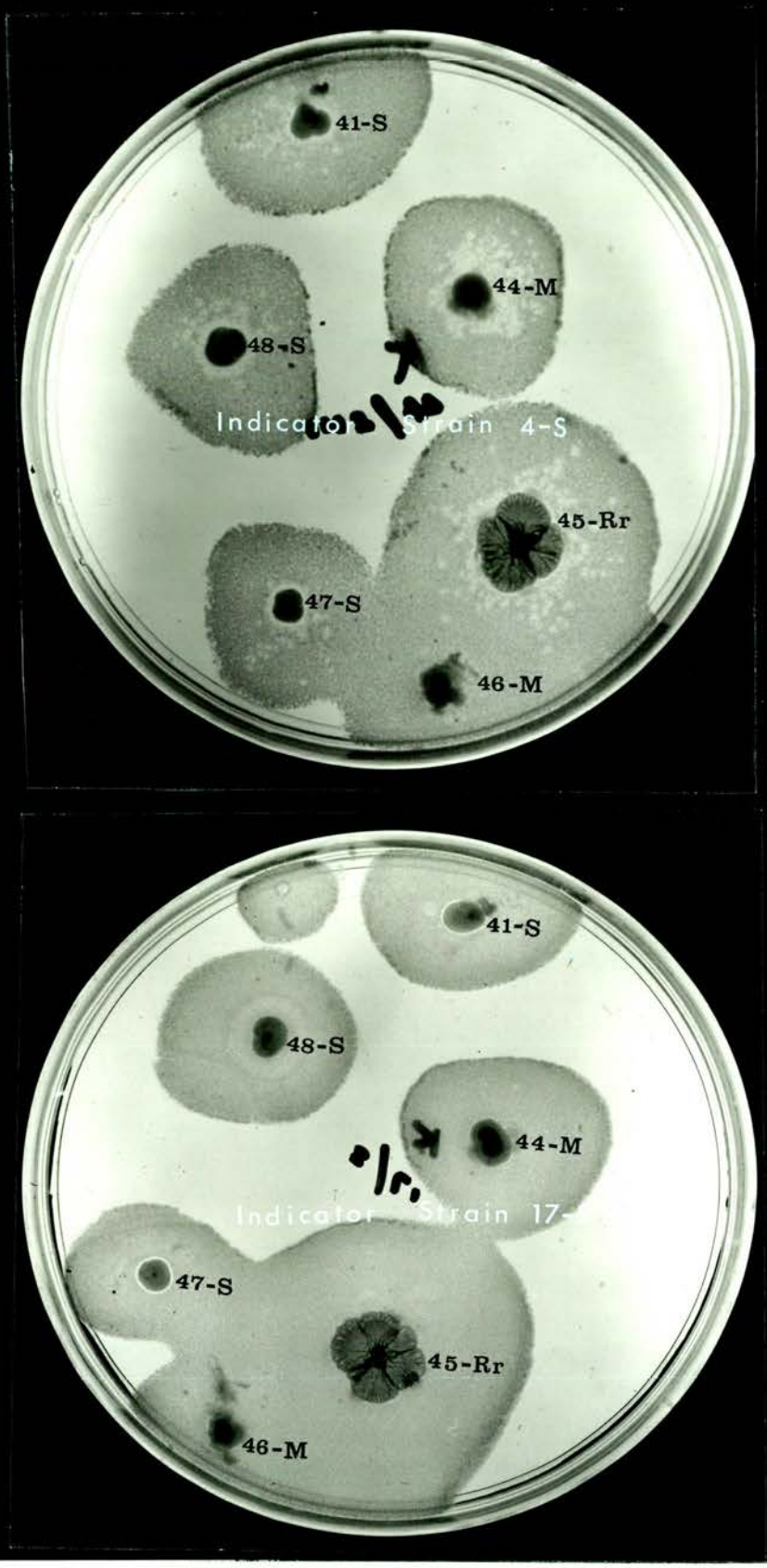


PLATE 25b

Plates 25 (a) & (b). The zones of inhibition produced by strains of Ps.pseudomallei (16 strains) when tested by Method 1 against 3 other strains (81, 27 and 15).
Note the wide zones of inhibition produced by strains 90 and 100 on indicator-plate lawned with strain 81.

Plate 26. Method 1: Ps.pseudomallei strains 34 and 35 unlike some of the others have produced wide zones of inhibition.



Plates 27 & 28. Strains of Ps.pseudomallei (strains 41-S, 44-M, 45 Rr, 46-M, 47-M & 48-S) tested by Method 2 against 2 other strains of the species. Phage plaques can be seen scattered around some of the zones of inhibition.

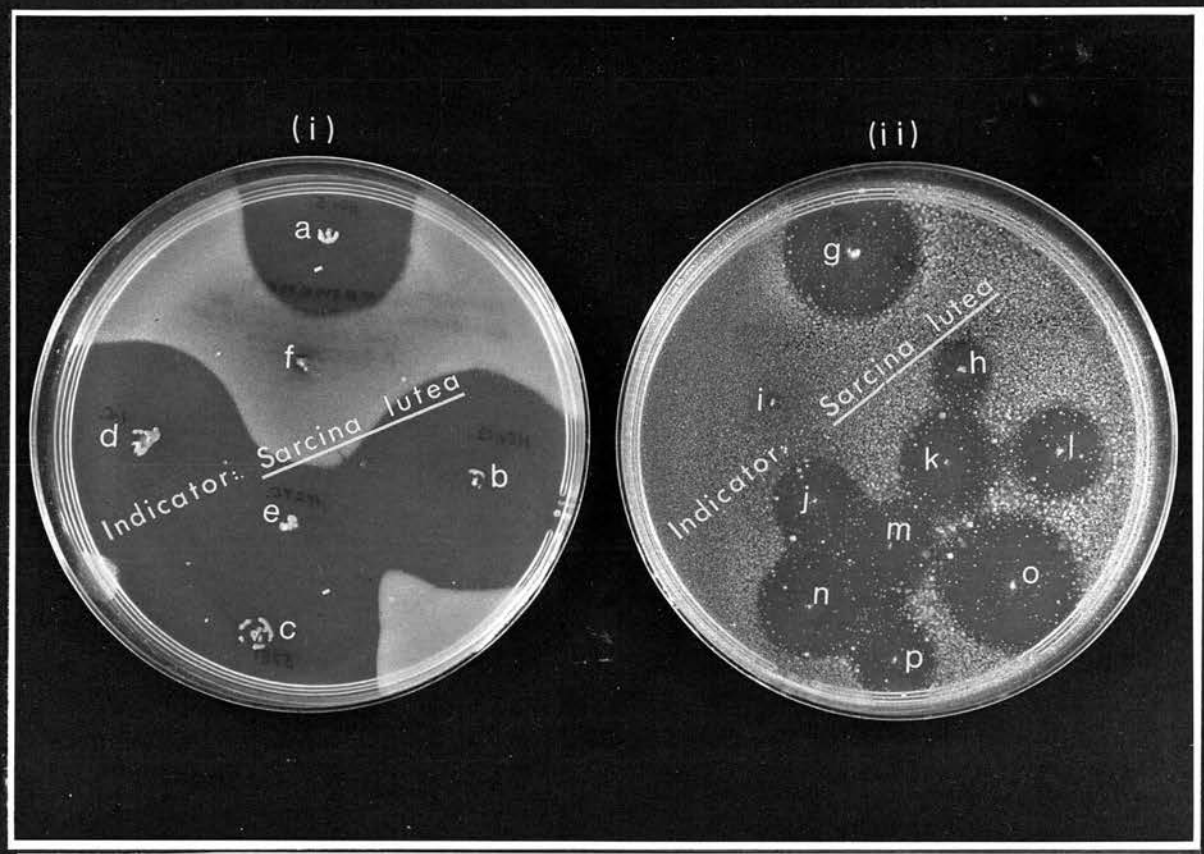


Plate 29, (i) & (ii). Zones of inhibition caused by 5 strains (a to e) of Ps.aeruginosa and 11 strains (f to p) of Ps.pseudomallei when tested by Method 2 on Sarcina lutea. (The "producer" strains were grown as 'spots' for 48 hours and were then scraped off before overlayering with the "indicator").

a = Ps.aeruginosa strain		i = Ps.pseudomallei strain	
b = " " "		j = " " "	
c = " " "		k = " " "	
d = " " "		l = " " "	
e = " " "		m = " " "	
f = Ps.pseudomallei "		n = " " "	
g = " " "		o = " " "	
h = " " "		p = " " "	

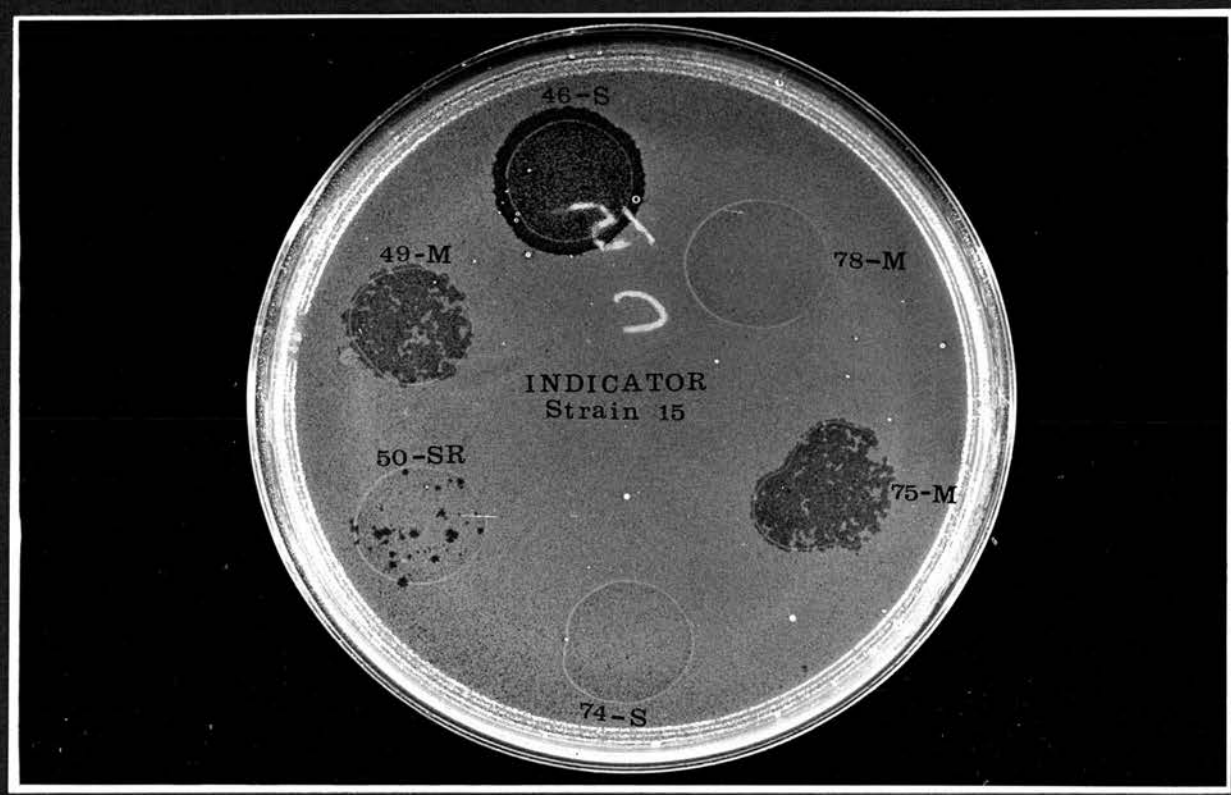
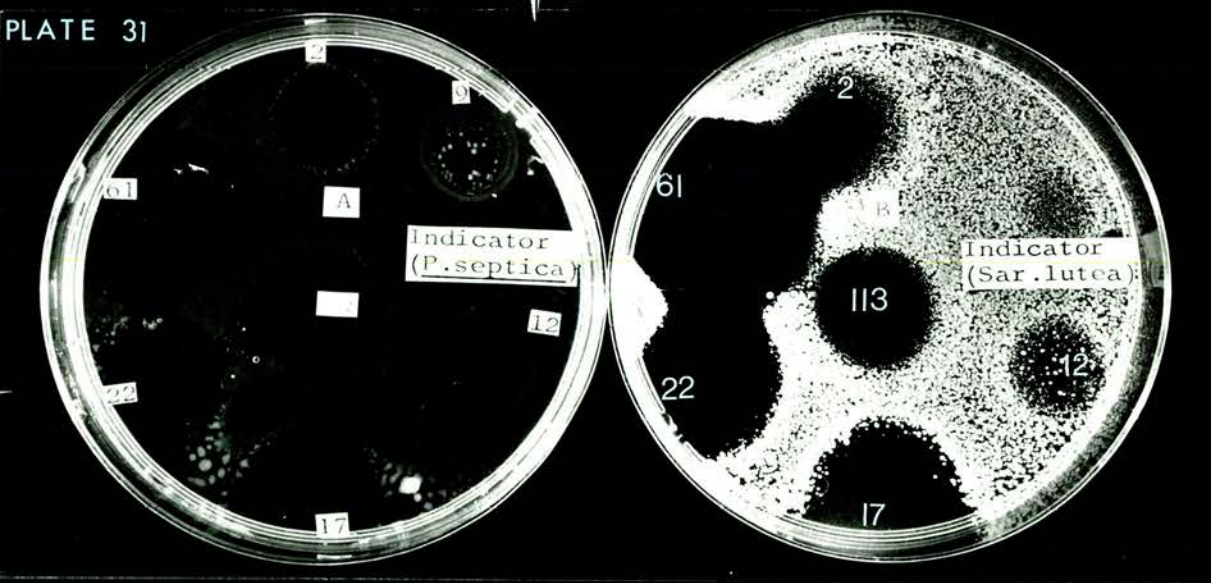
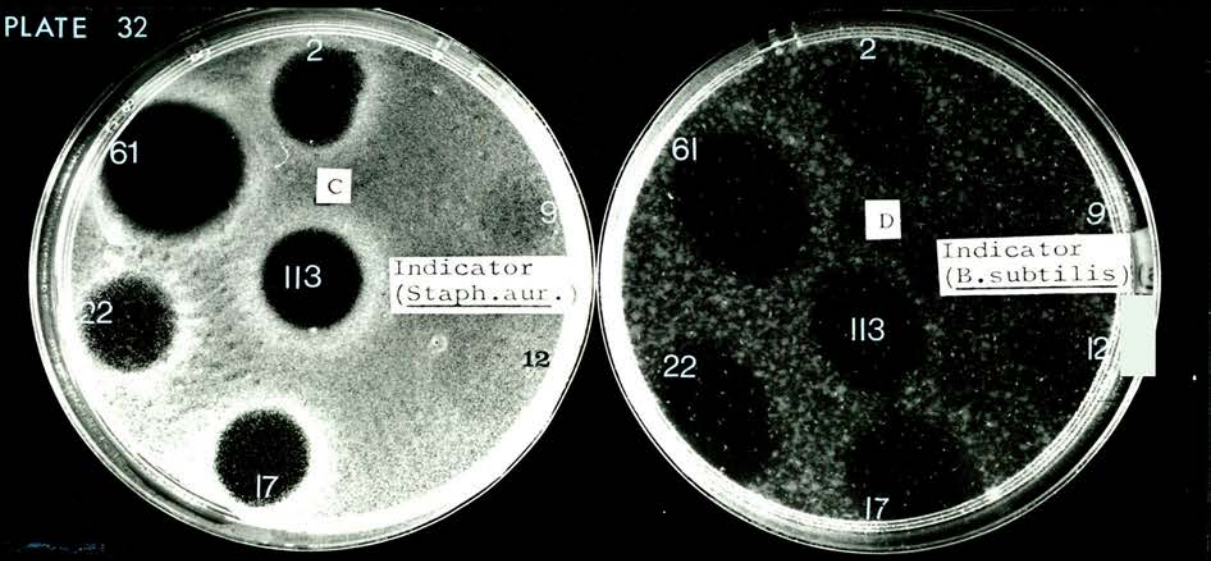
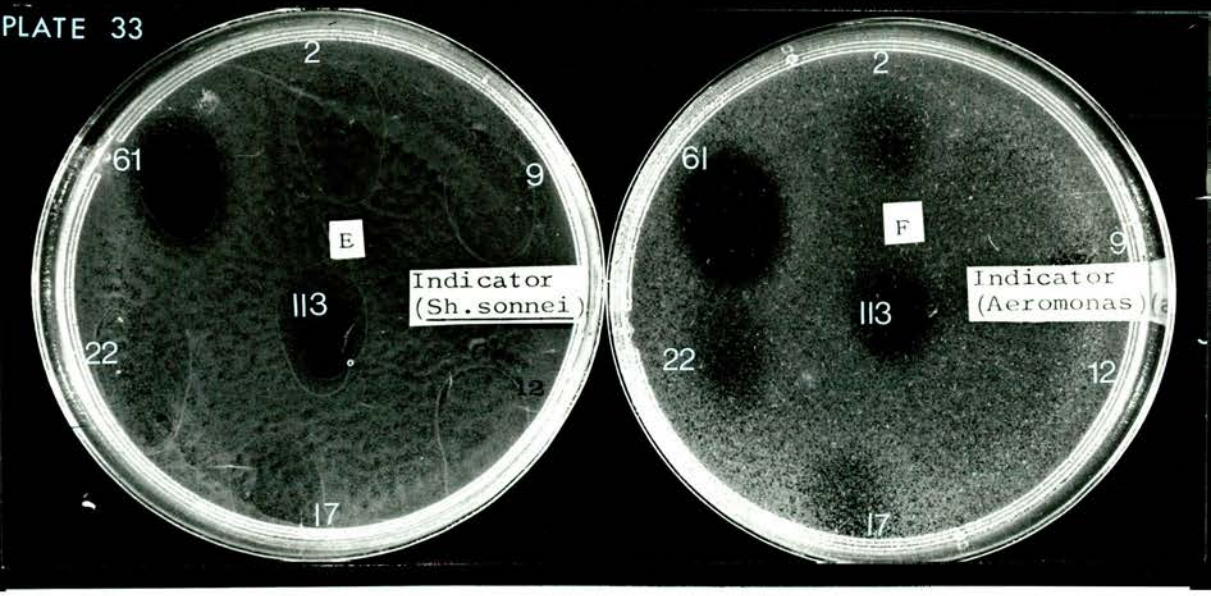


Plate 30. Discrete phage plaques or zones of confluent inhibition produced by sterile broth culture supernates of Ps.pseudomallei (strains 46-S, 49-M, 50-SR, 74-S, 75-M & 78-M) tested by Method 3 on a lawn of Ps.pseudomallei strain.







Plates 31, 32 & 33. Zones of confluent inhibition (weak or strong) produced by 7 strains of Ps.pseudomallei (strains 2, 9, 12, 61, 113, 17 and 22) when tested by Method 3 against some unrelated bacteria (Past.septica, Sarcina lutea, Staph. aureus, B.subtilis, Sh.sonnei and Aeromonas hydrophila).

PLATE 35

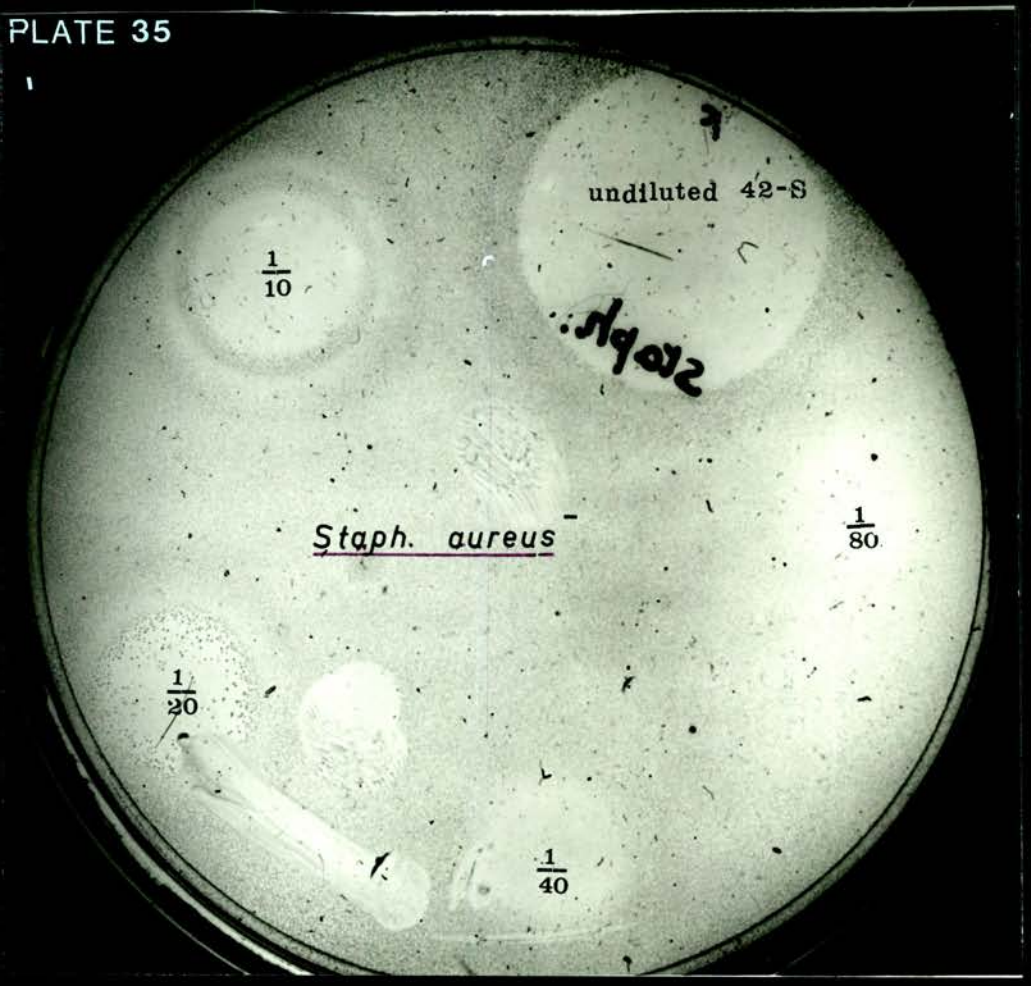


Plate 35. Dilutions of sterile broth culture supernate of Ps.pseudomallei strain 42-S tested by Method 3 on a lawn of Staph.aureus.

PLATE 34

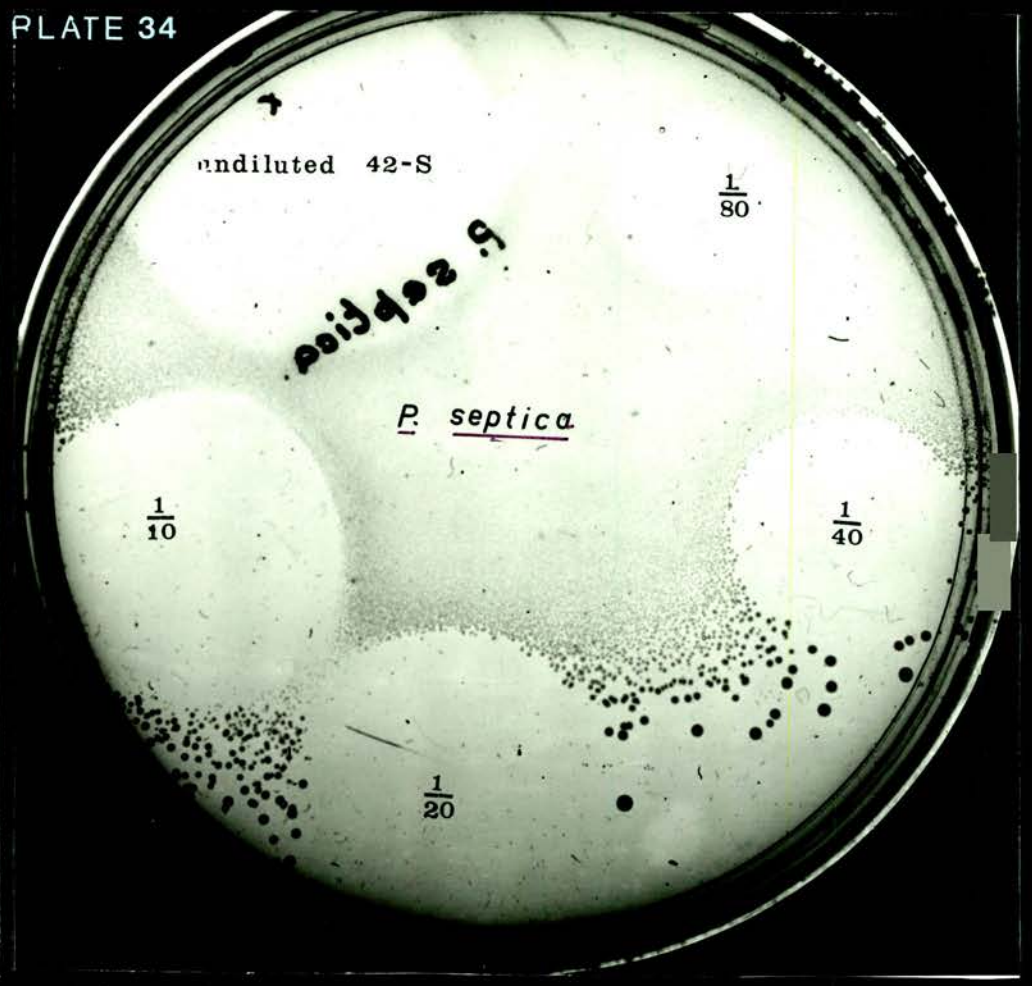


Plate 34. Dilutions of a sterile broth culture supernate of Ps.pseudomallei strain 42-S tested by Method 3 on a lawn of Past.septica.

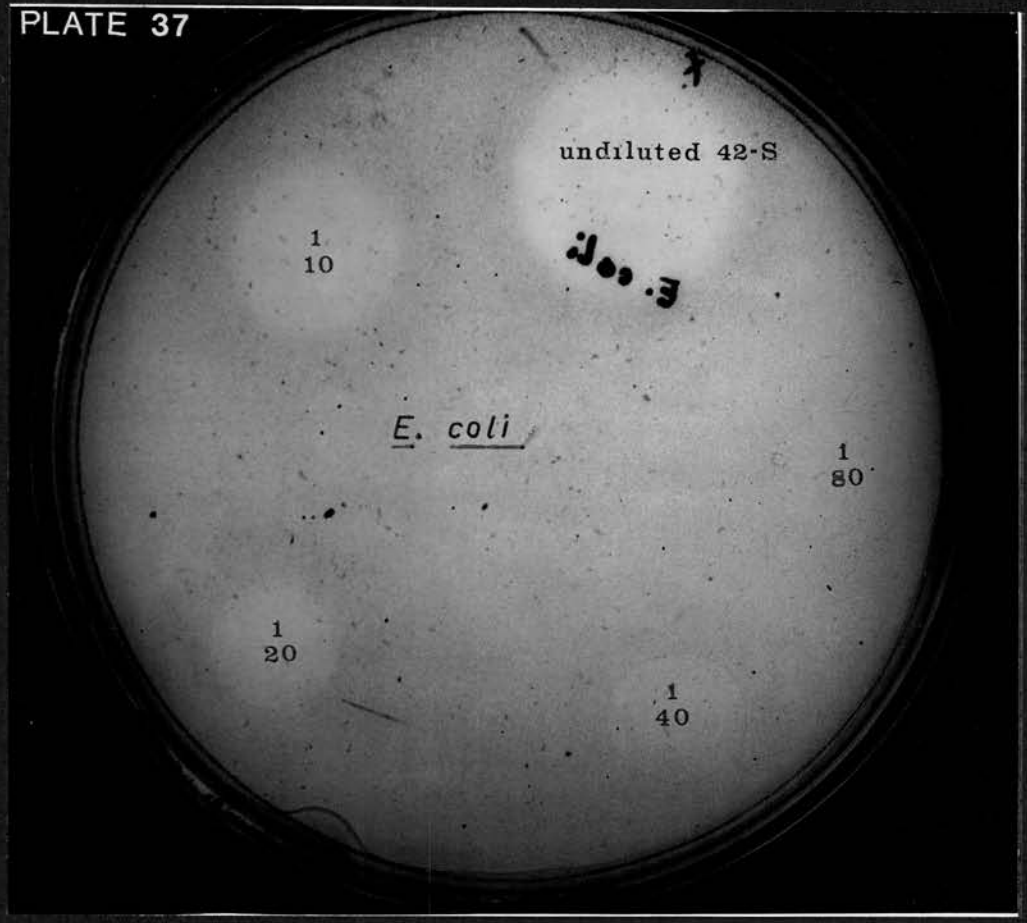


Plate 37. Dilutions of a sterile broth culture supernate of Ps.pseudomallei strains 42-S tested by Method 3 on a lawn of E.coli.

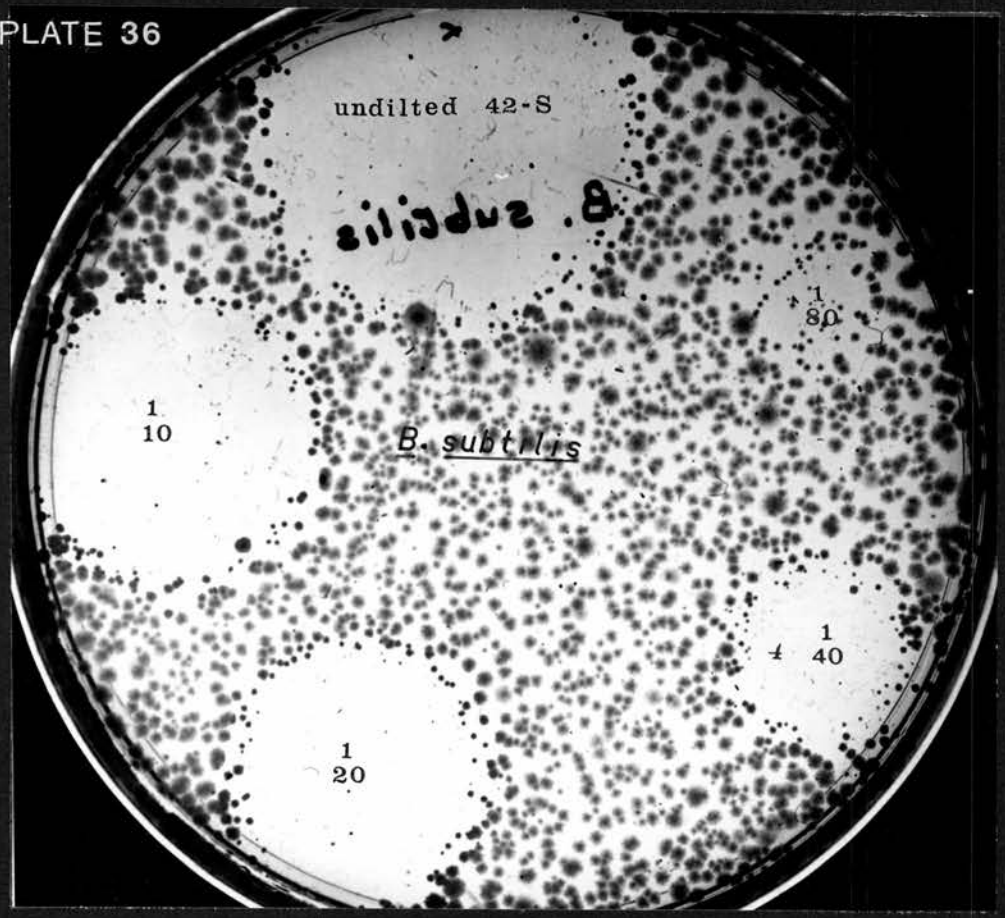


Plate 36. Dilutions of a sterile broth culture supernate of Ps.pseudomallei strain 42-S tested by Method 3 on a lawn of B.subtilis.

PLATE 38

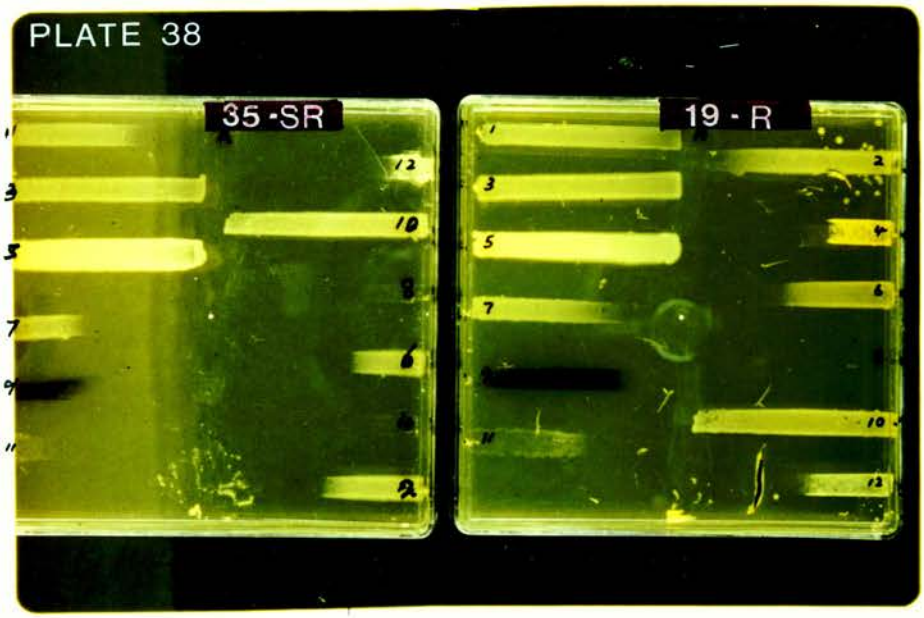


Plate 38. The "streak" method to demonstrate the inhibitory effects of 2 strains of Ps.pseudomallei on:- (1) S.typhi; (2) E.coli; (3) Kl.pneumoniae; (4) Sarcina lutea; (5) Kl.aerogenes; (6) Aeromonas hydrophila; (7) Shig. sonnei; (8) Past.septica; (9) Chromobact.violaceum; (10) Serratia marcesens; (11) Enterobacter cloacae; (12) Past.haemolytica.

PLATE 39

Plate 39. The inhibitory effects of Ps.pseudomallei strains 2-M; 86-S and 92-S on Ps.aeruginosa and unrelated bacteria - 1. Staph.aureus; 2. Ps.aeruginosa 3. Aeromonas hydrophila; 4. Kl.aerogenes; 5. Past.septica.

PLATE 40

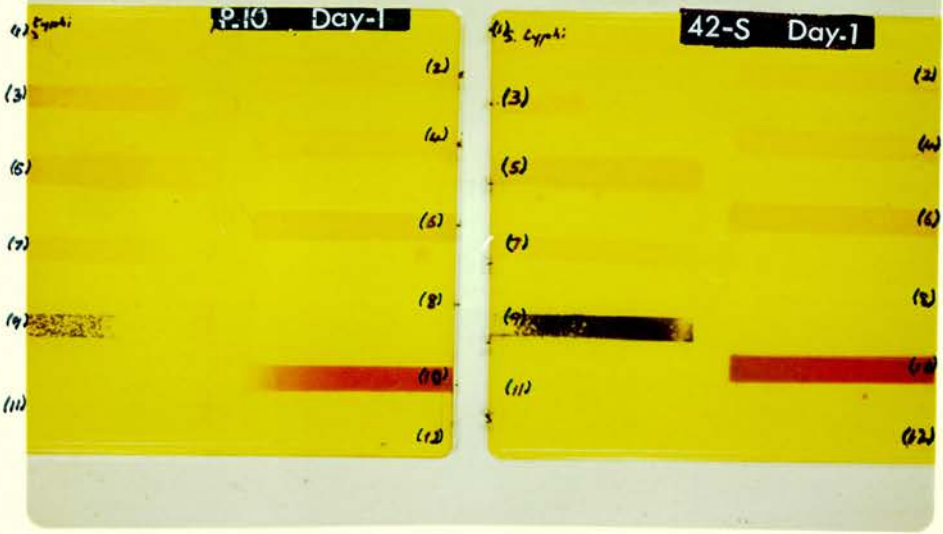


Plate 40. Inhibitory effects seen when strains of other bacteria*** were 'cross-streaked' on plates in which Ps.aeruginosa (strain P.10) or Ps.pseudomallei (strain no. 42) had been grown for 24 hours.

PLATE 41

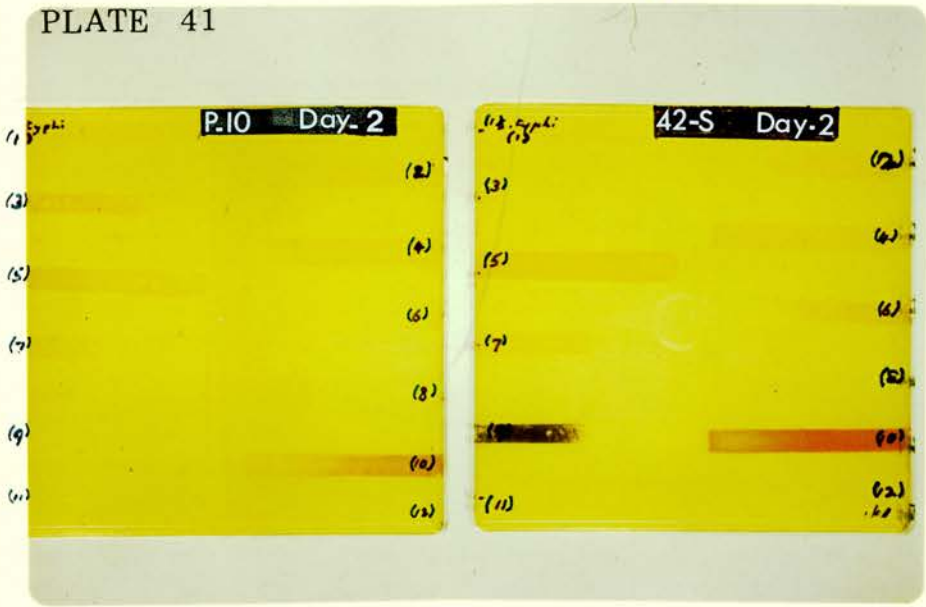


Plate 41. Inhibitory effects seen when strains of other bacteria*** were 'cross-streaked' on plates in which Ps.aeruginosa (strain P.10) or Ps.pseudomallei (strain no. 42) had been grown for 48 hours.

***
(1) S.typhi
(2) E.coli
(3) Sarcina lutea
(4) Kl.pneumoniae
(5) Kl.aerogenes
(6) Aeromonas hydrophila
(7) Sh.sonnei
(8) Past.septica
(9) Chromobact.violaceum
(10) Serratia marcesens
(11) Enterobact.cloacae
(12) Past.haemolytica

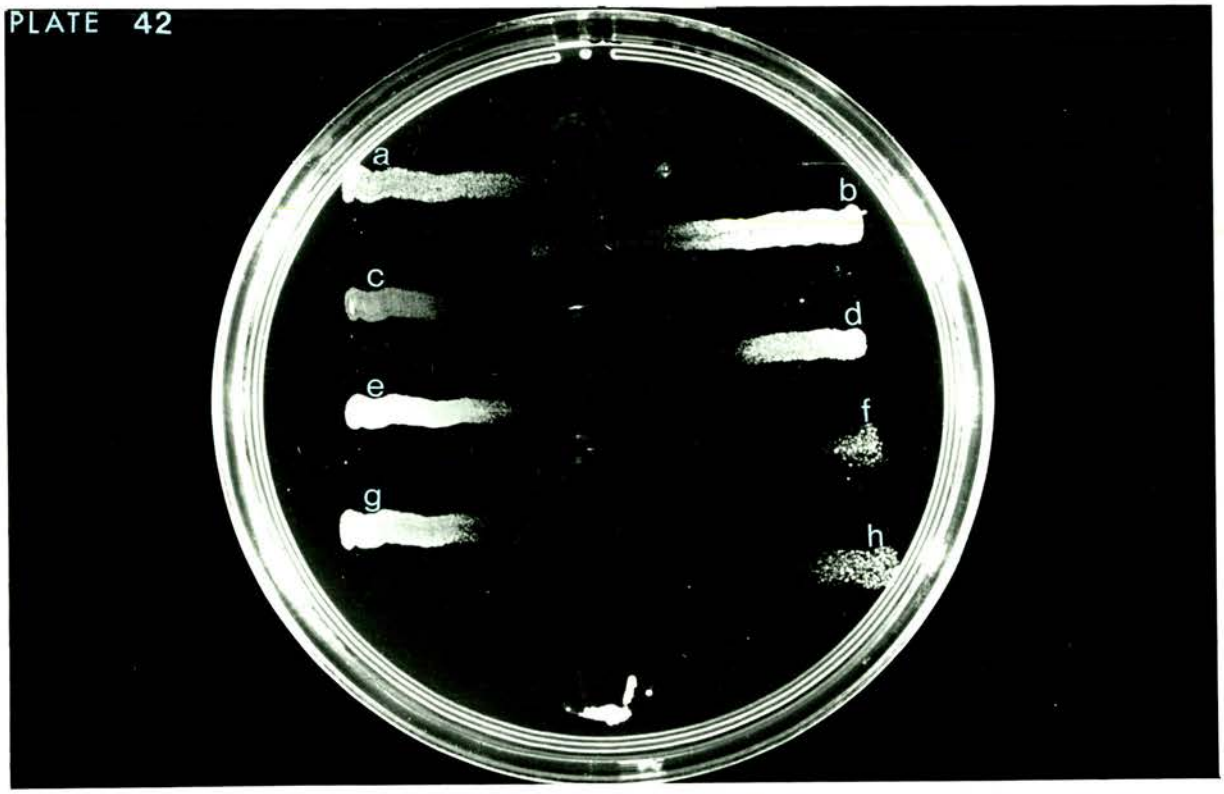


Plate 42. The uniform inhibitions caused by a "mucoid" strain of Ps.pseudomallei (strain 15-M) on 8 other strains of Ps.pseudomallei selected at random.

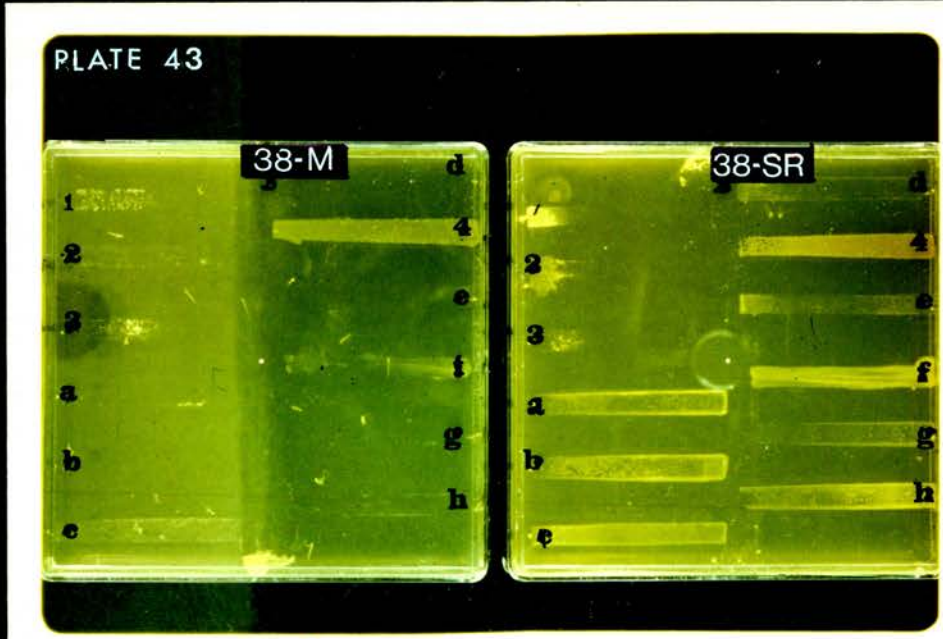


Plate 43. Inhibitory effects of 2 variants ("M" & "S") of Ps.pseudomallei strain 38 on other strains* including 3 unrelated bacteria.

1.Staph.aureus	a.Ps.pseudomallei - 112	e.Ps.pseudomallei - 4
2.B.subtilis	b.Ps.pseudomallei - 114	f.Ps.pseudomallei - 6
3.Past.septica	c.Ps.pseudomallei - 38.M	g.Ps.pseudomallei - 8
4.Ps.aeruginosa	d.Ps pseudomallei - 38-SR	h.Ps.pseudomallei -15

PLATE 44

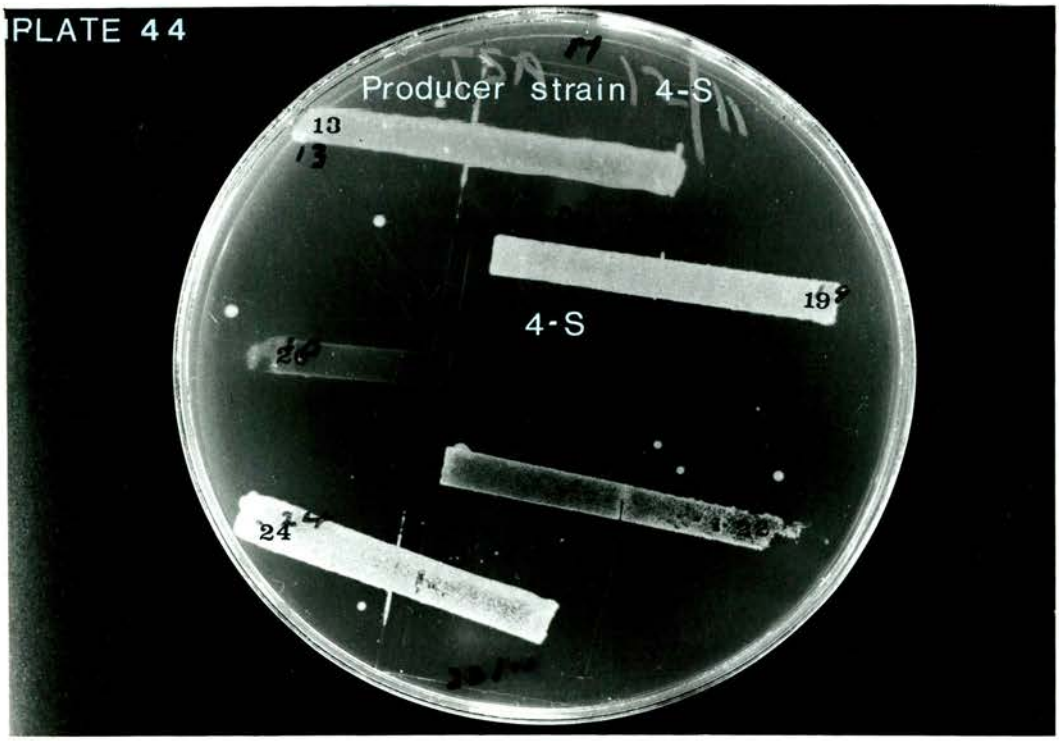
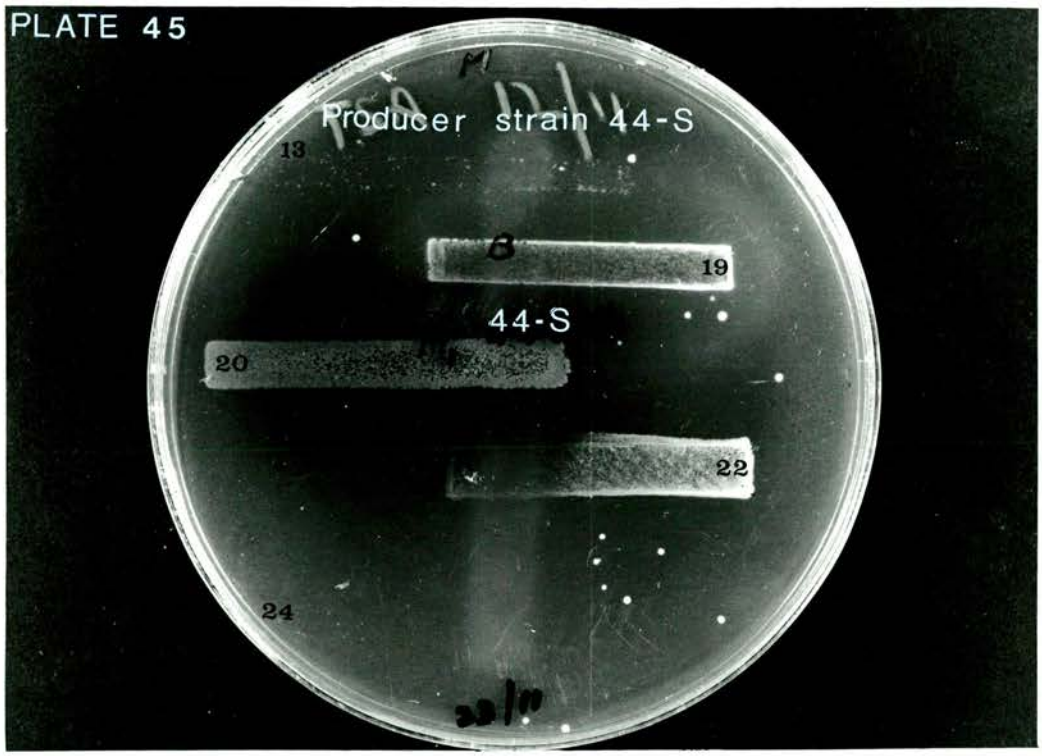


PLATE 45



Plates 44 & 45. The "gamma" type inhibitory effects caused by 2 strains of Ps.pseudomallei (strains 4-S and 44-S) on an identical set of Ps. pseudomallei strains used as indicators.

PLATE 46

Plate 46. The inhibitory effects of Ps.pseudomallei strains 35 and 110 which had been irradiated for 3 minutes during growth. Although some or all of the 7 strains of Ps.pseudomallei (2, 4, 27, 33, 74, 79 and 81) cross-streaked against the two producer strains, have been inhibited, they have also shown resistant growth on and adjacent to the site of each of the 'producer' streaks.

PLATE 47

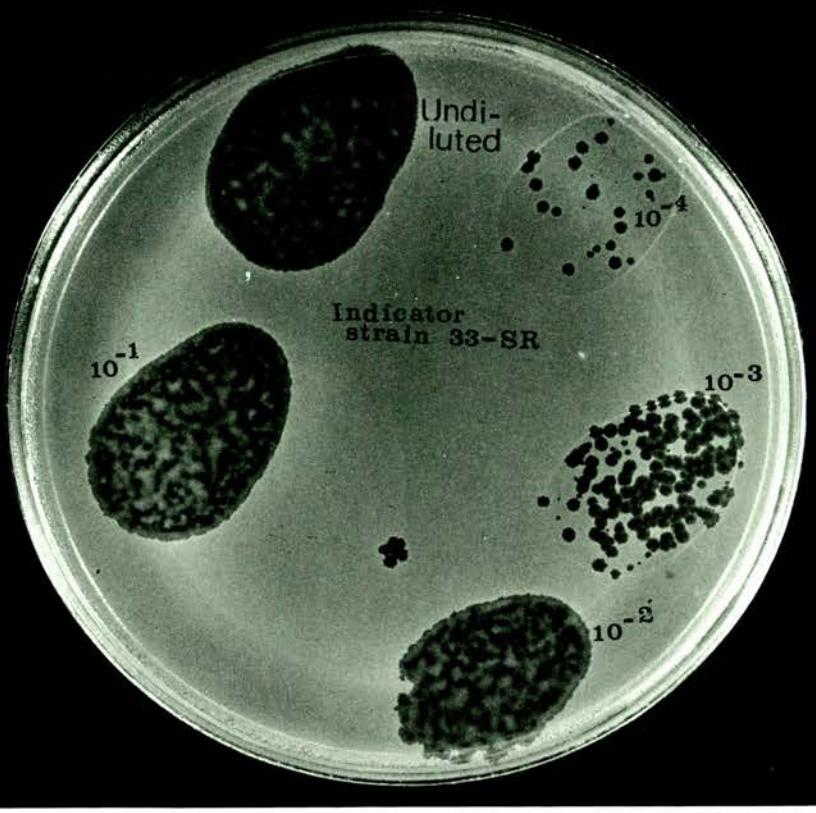
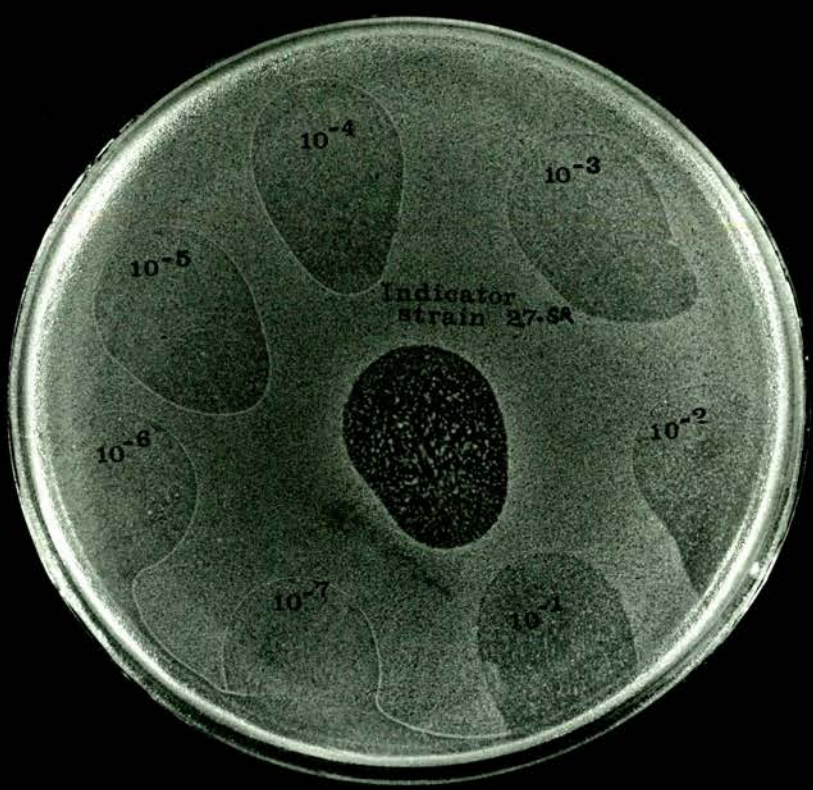


PLATE 48



Plates 47 & 48. Dilutions of Fraction-1 preparation of Ps.pseudomallei strain 35 tested by Method 3 on lawns of Ps.pseudomallei strains 33SR and 27-SR
Note the weak zone of inhibition and the failure to produce phage plaques on the plate lawned with strain 15.

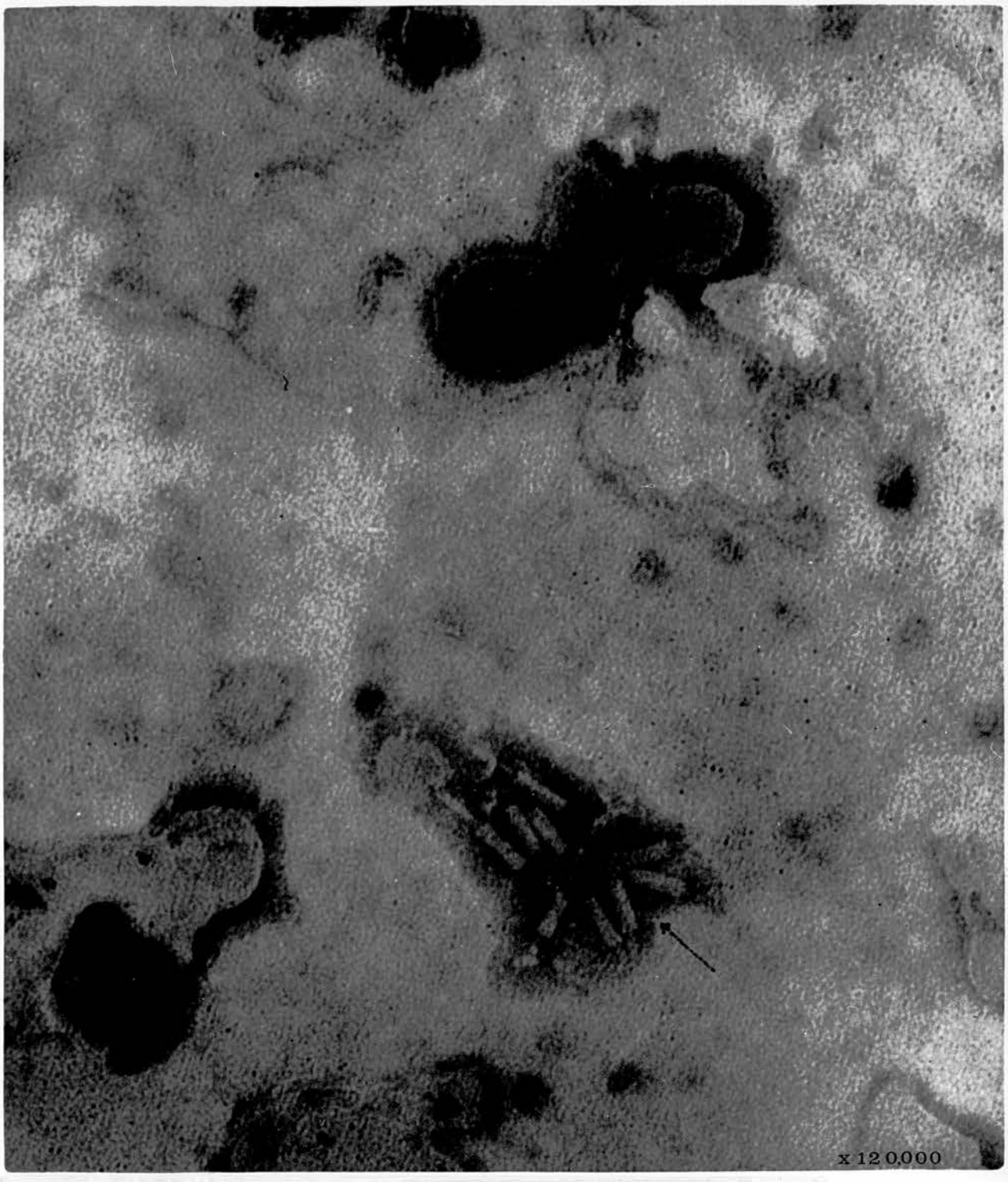


Plate 49. Structures resembling the tails of bacteriophages found in Fraction 1 specimens from Ps.pseudomallei strain 35-SR.

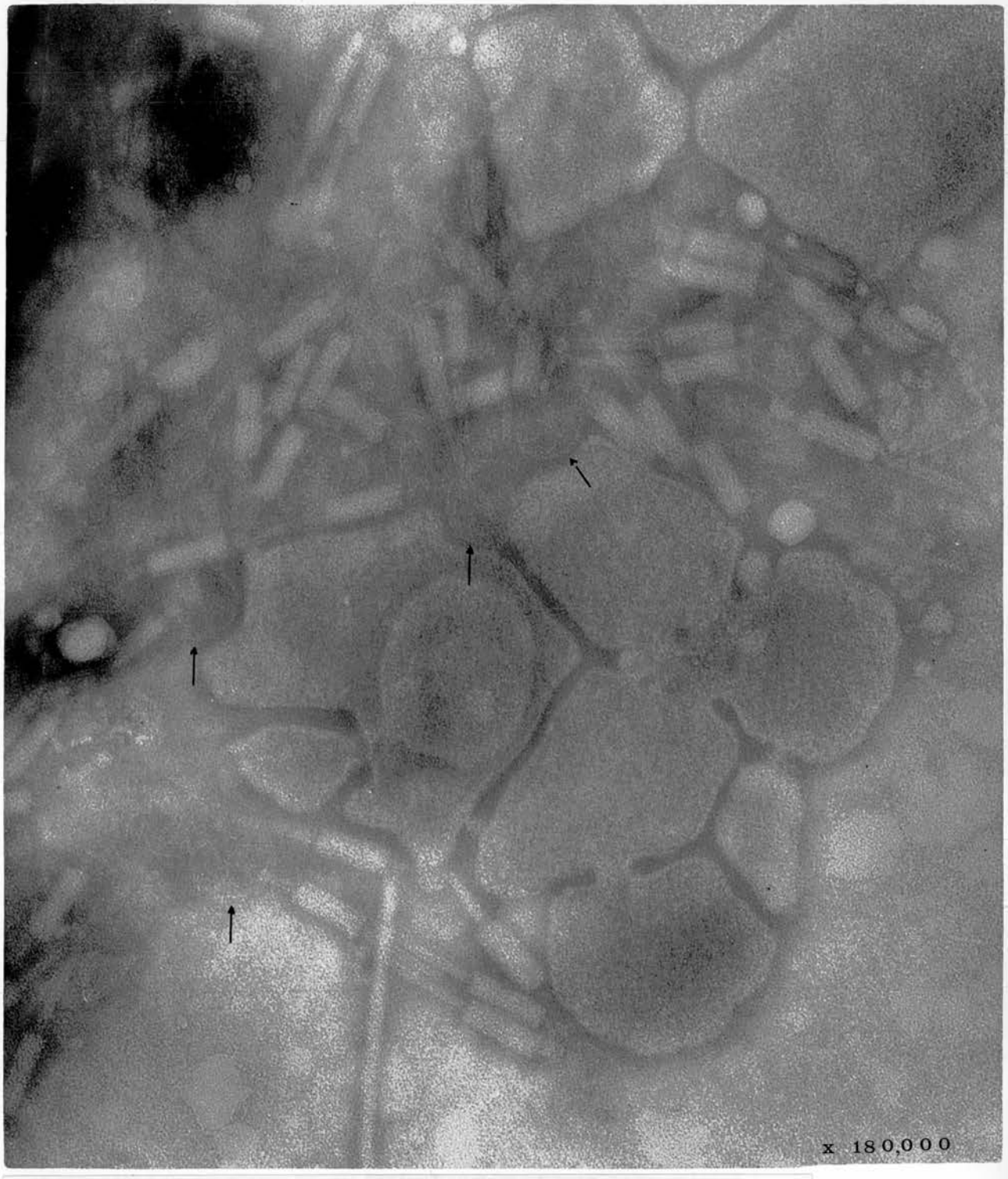
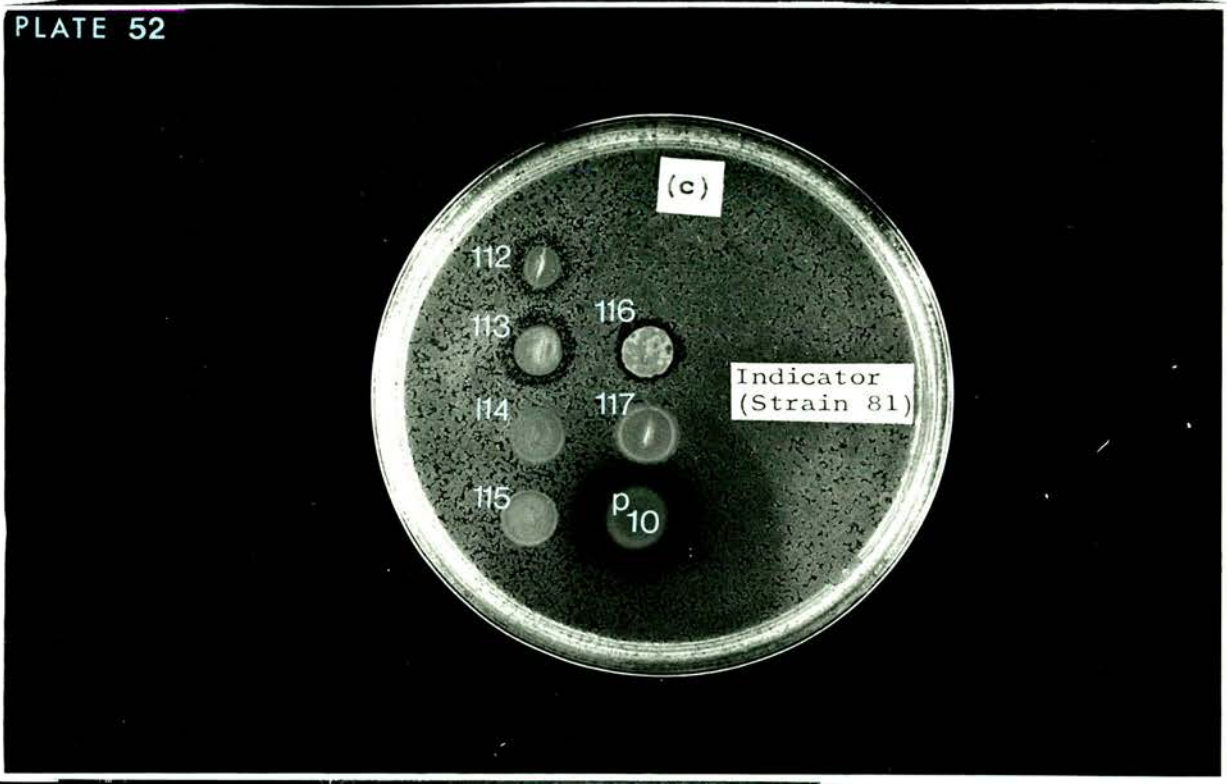


Plate 50. Structures resembling the tails of bacteriophages found in Fraction 1 specimens from Ps.pseudomallei strain 46. Note the faint outlines (arrowed) of what appear to be empty phage heads.

PLATE 51

PLATE 52



Plates 51 and 52. A strain of Ps.aeruginosa (strain P.10) tested alongside 7 strains of Ps.pseudomallei (strains 112, 113, 114, 115, 116 and 117) on 3 strains of Ps.pseudomallei (strains 15, 27 and 81). Unlike the strains of Ps.pseudomallei, the Ps.aeruginosa strain has inhibited all 3 indicators and has produced relatively wide groups of inhibition.

PLATE 54

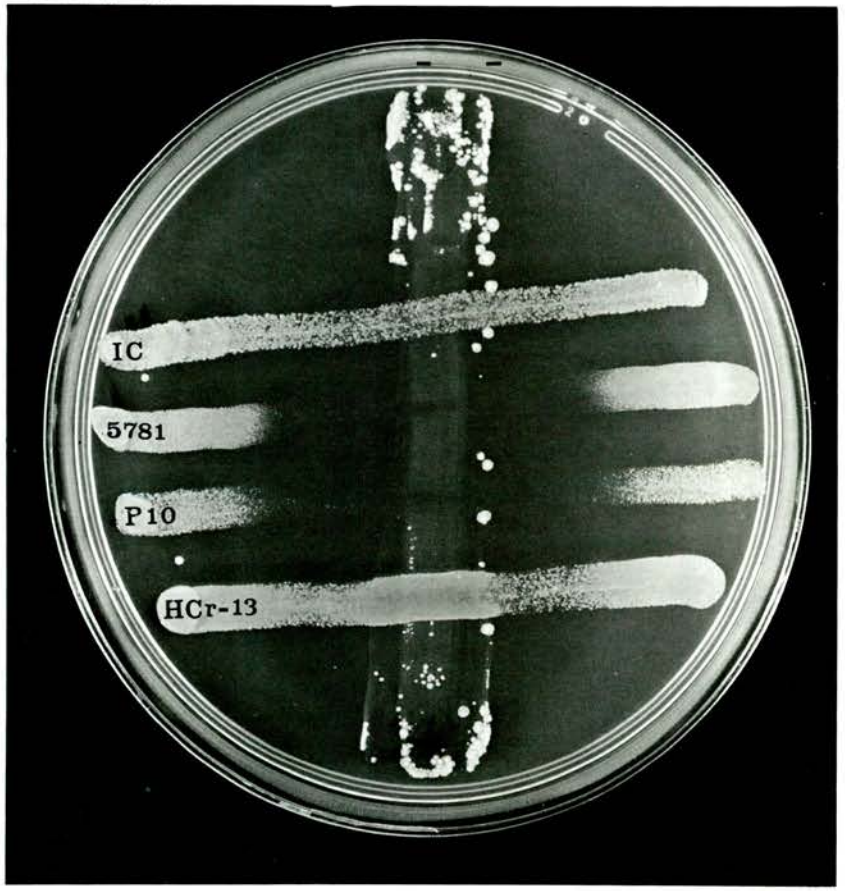


Plate 54. The inhibitory effects of Ps.aeruginosa strain "HCr-5" towards 4 other strains of Ps.aeruginosa (strains P.10; NCTC 5781; "HCr-13" and "I-C").

PLATE 53

Plate 53. The uniform zones of intense inhibition caused by Ps.aeruginosa strain"HCr-5" on 4 strains of Ps.pseudomallei picked at random.

PLATE 55

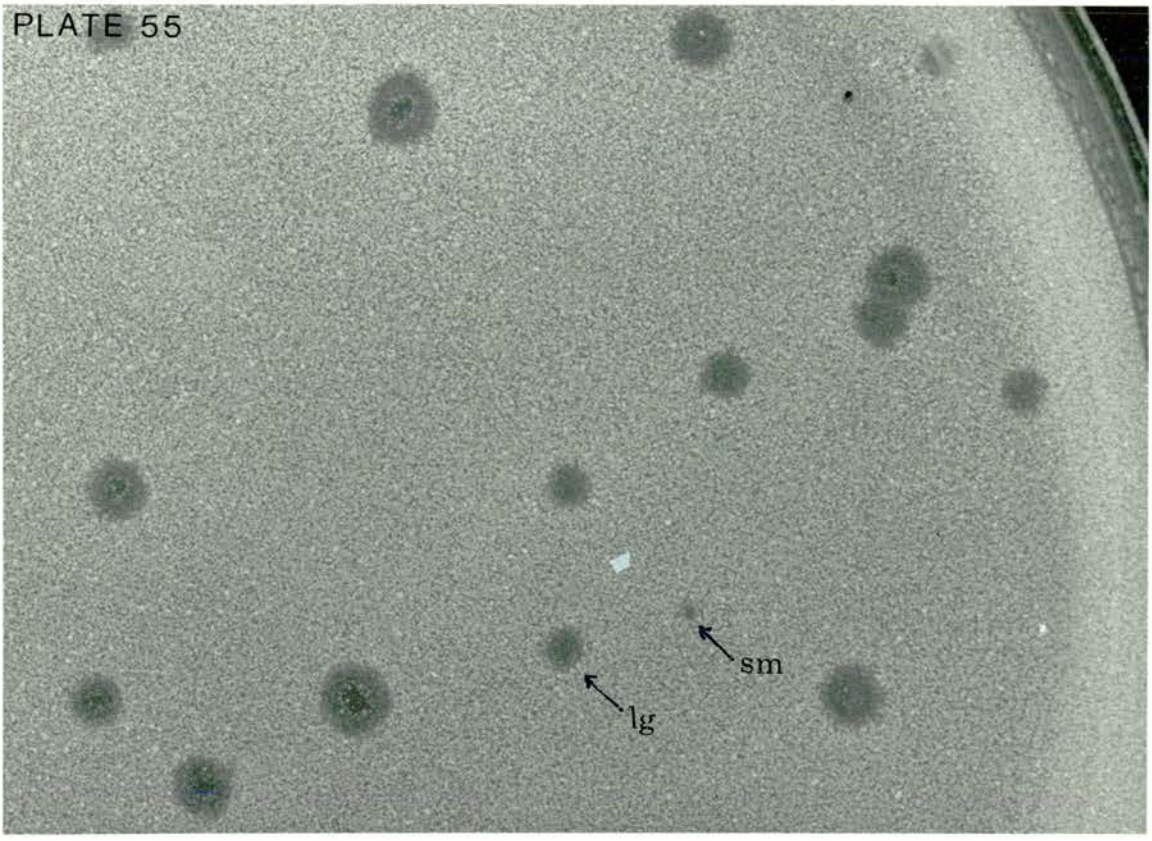
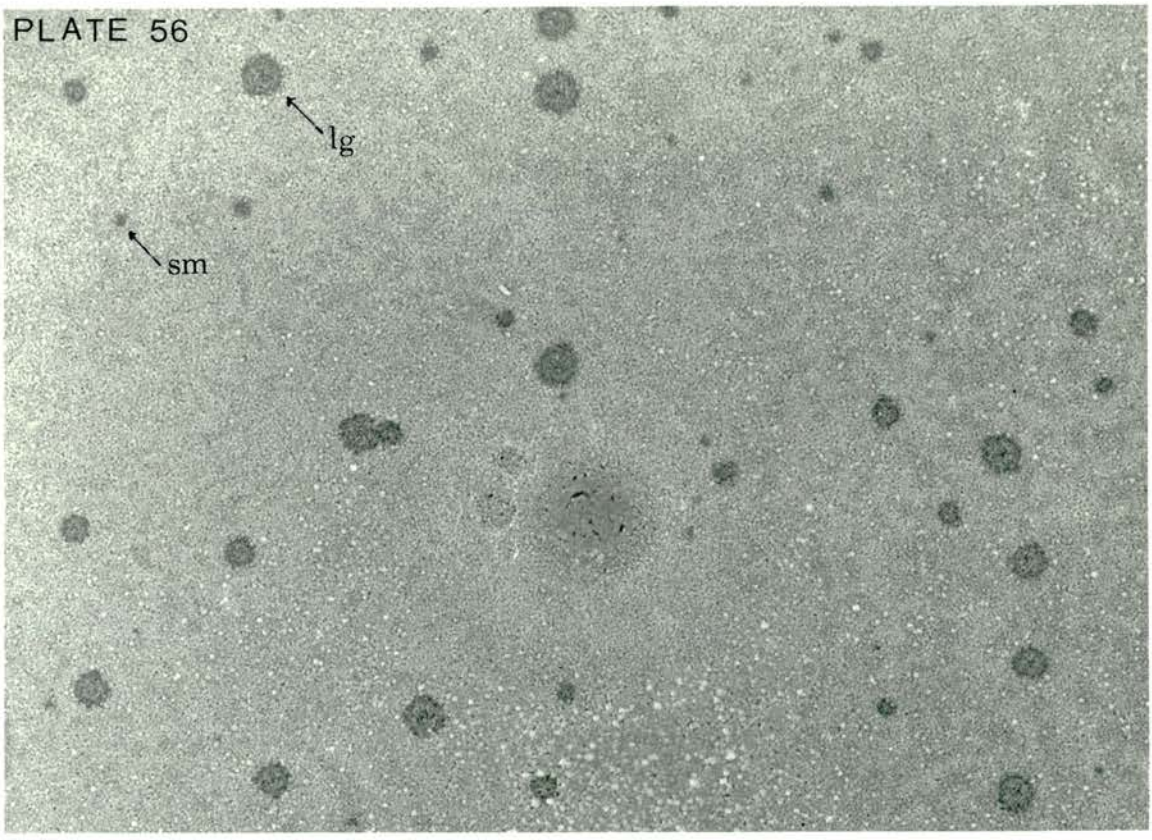


PLATE 56



Plates 55 and 56. The large (lg.) and small (sm.) phage plaques by purified phage preparations. Note also the secondary growth arising in the centre of some of the plaques.

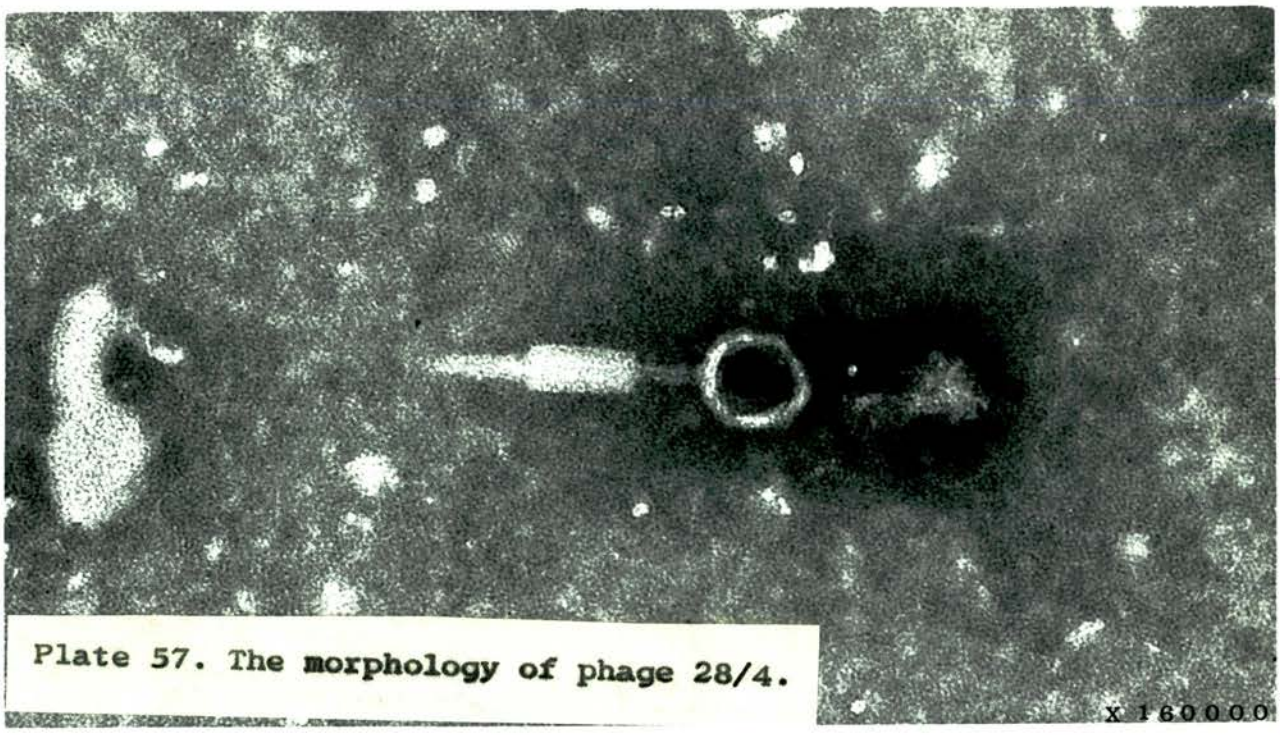


Plate 57. The morphology of phage 28/4.

Plate 58. Phage components (phage heads and tails) seen with intact phages in a preparation of phage 75/2. Note also the displacement of the contractile sheath (a) towards the tail-end.

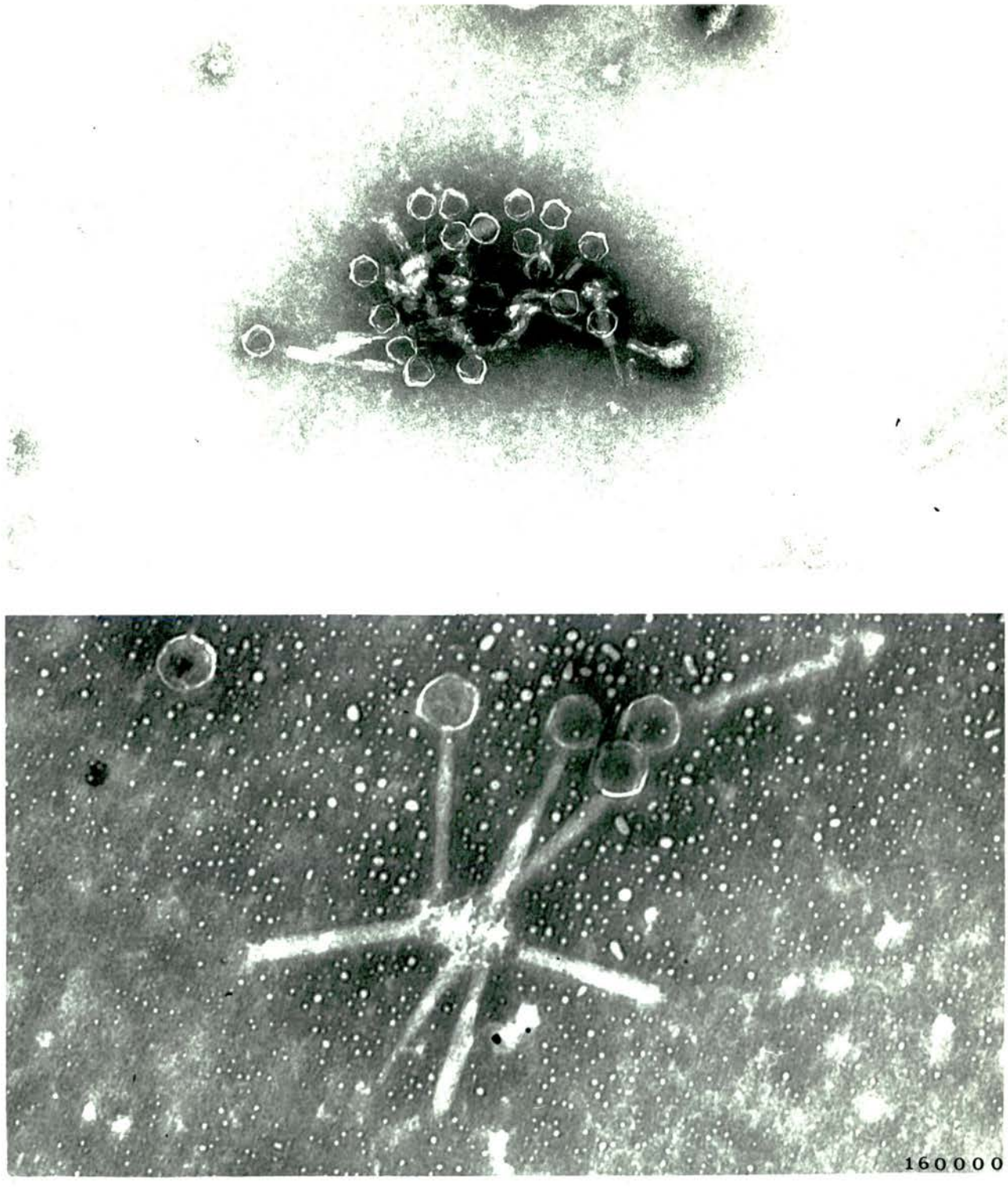
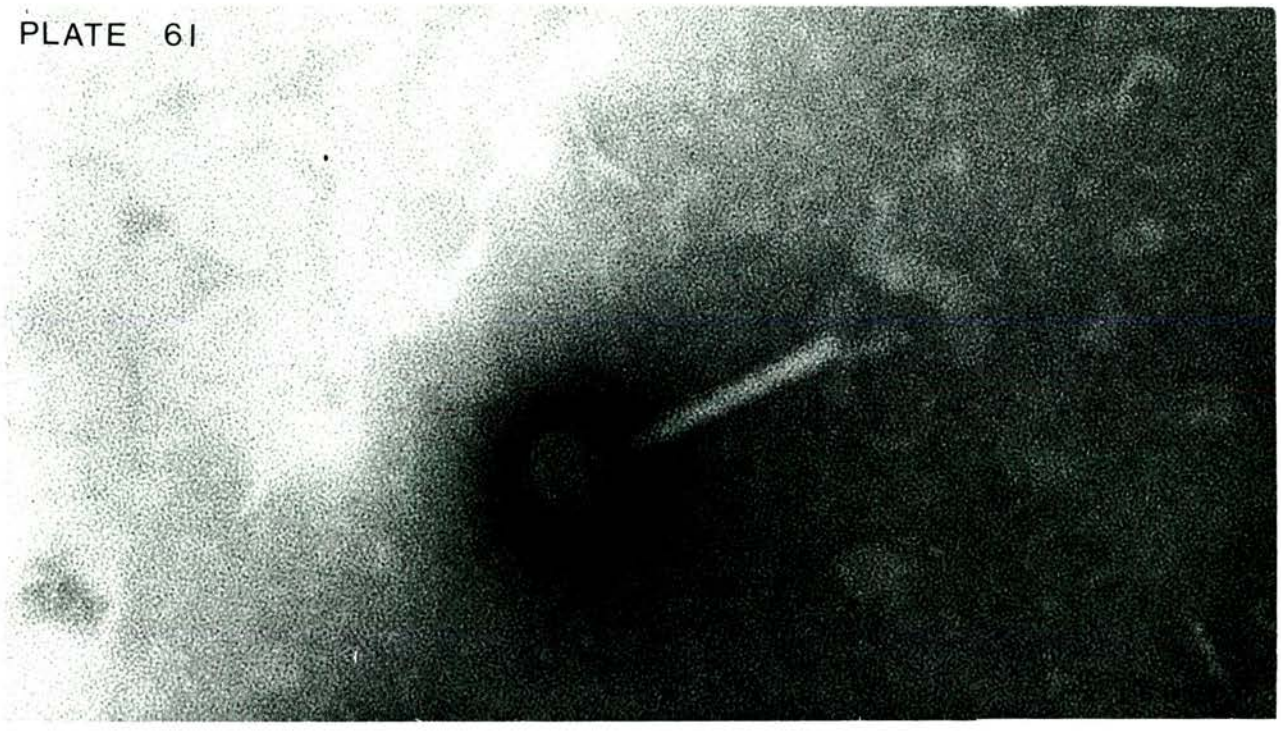


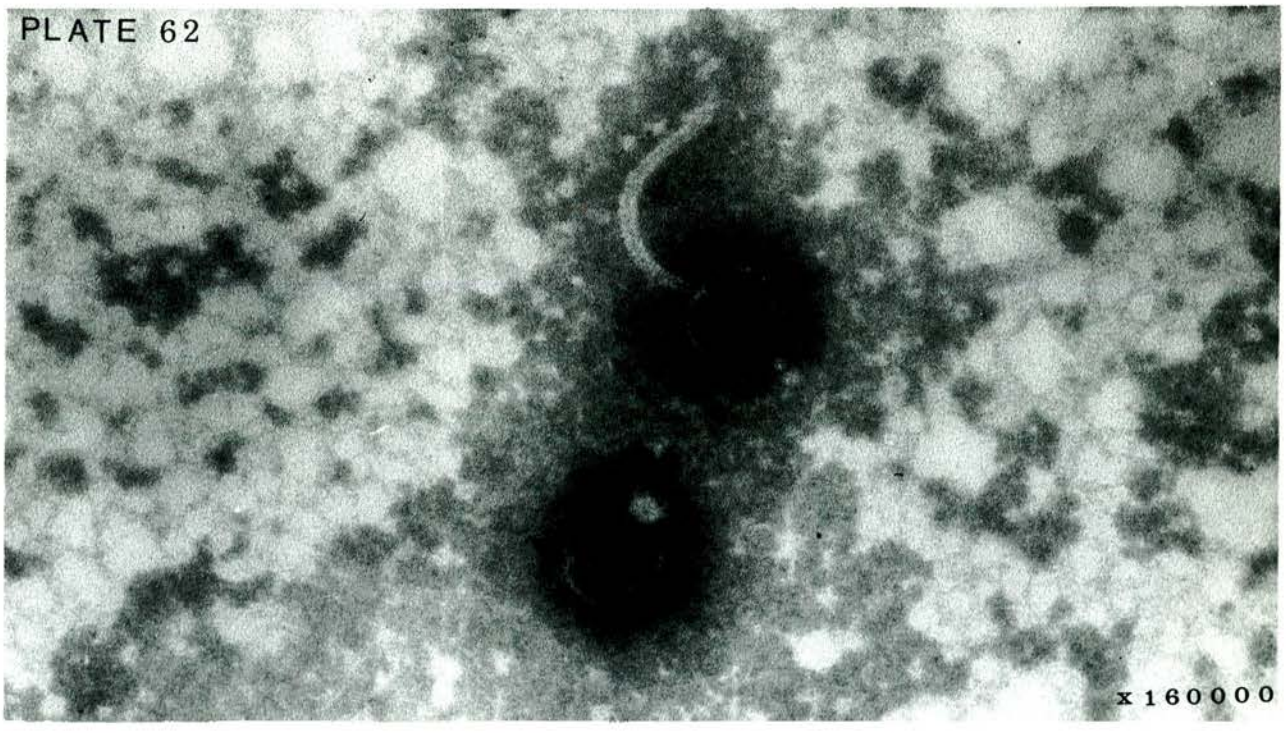
Plate 59. Phage components (heads and tails) found alongside a few intact phage particles in a preparation of phage 49/2.

Plate 60. Morphology of intact phage particles and phage-tails in a preparation of phage 18/2.

Plate 61. Morphology of phage 18/2.

Plate 62. Morphology of phage 46/2.





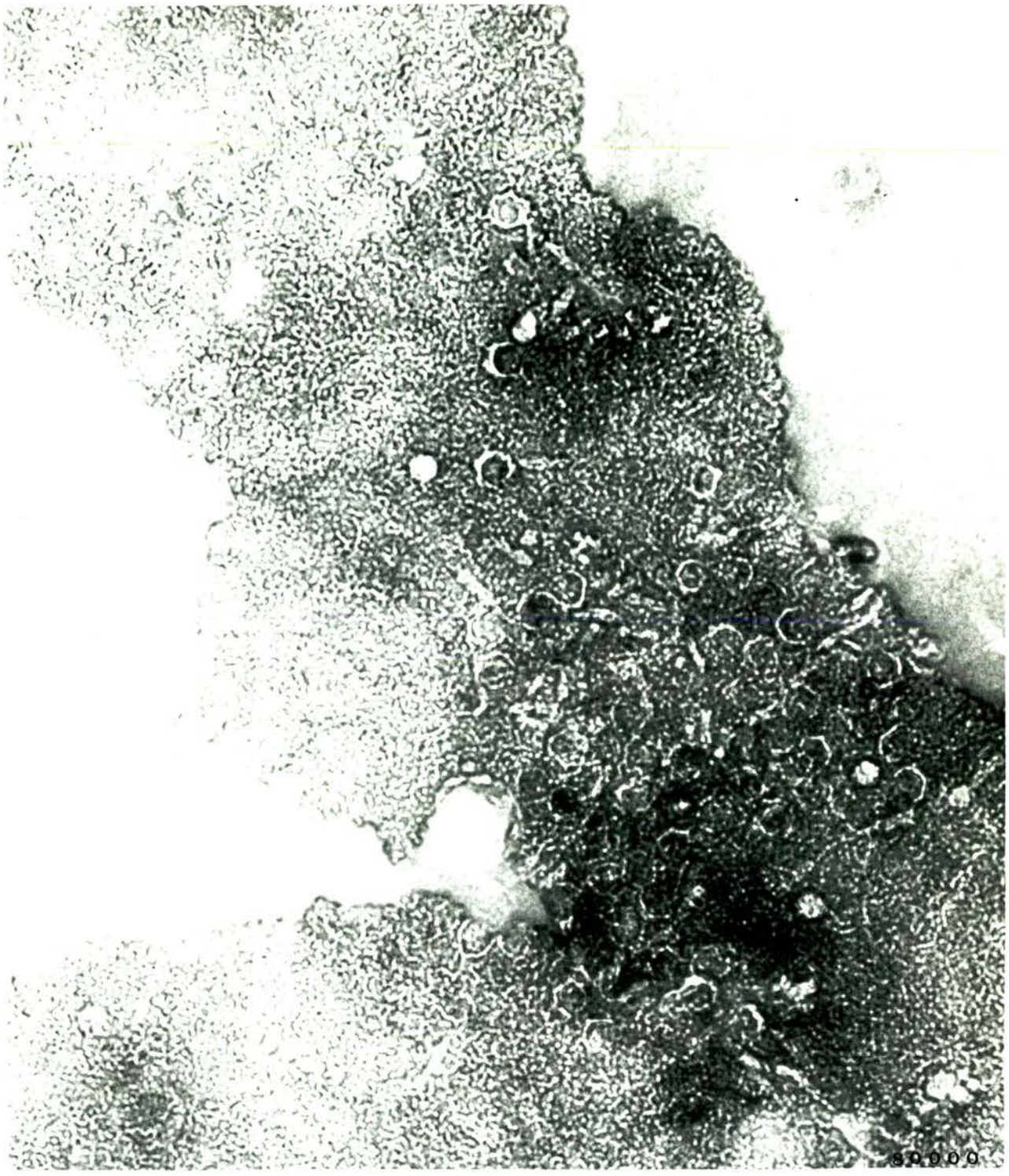
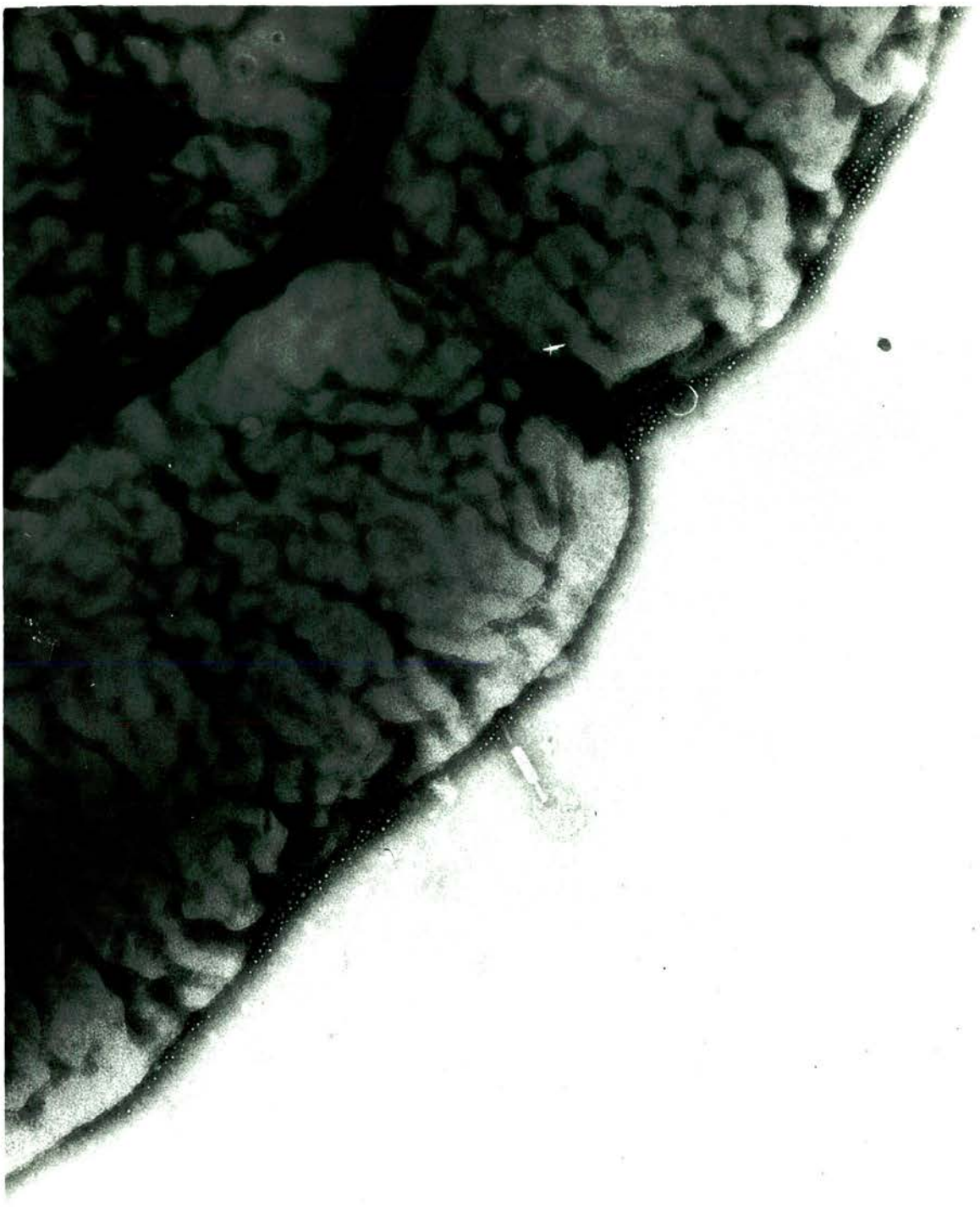


Plate 63. Particles seen in a preparation of phage 116/33.



X 60 000

Plate 64. Phage particle with coiled sheath seen attached to a host cell.

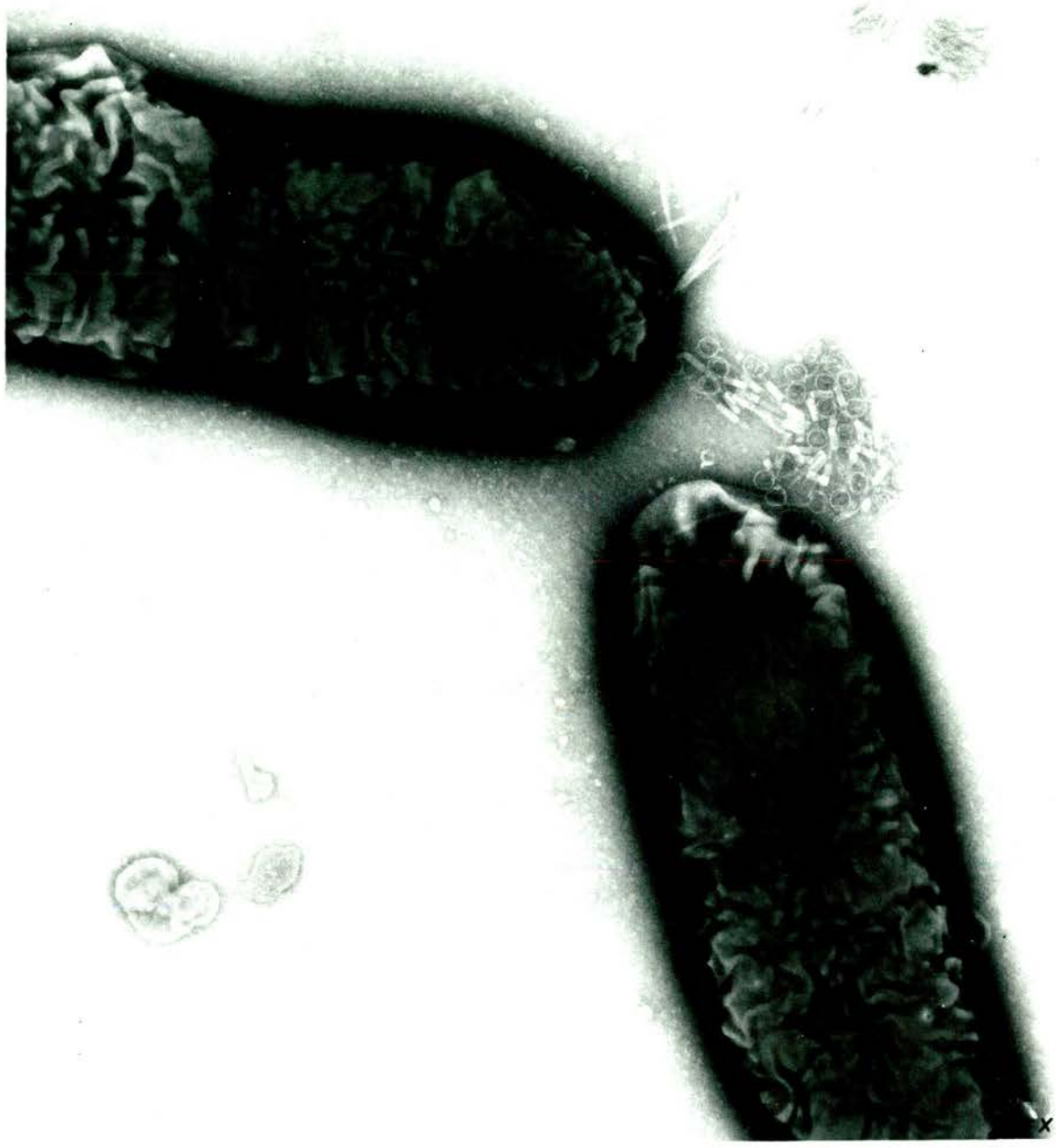


Plate 65. Cluster of unattached phage particles of phage 75/2.

Plate 66. Phage particle with uncoiled sheath seen attached to a host cell.

PLATE 67

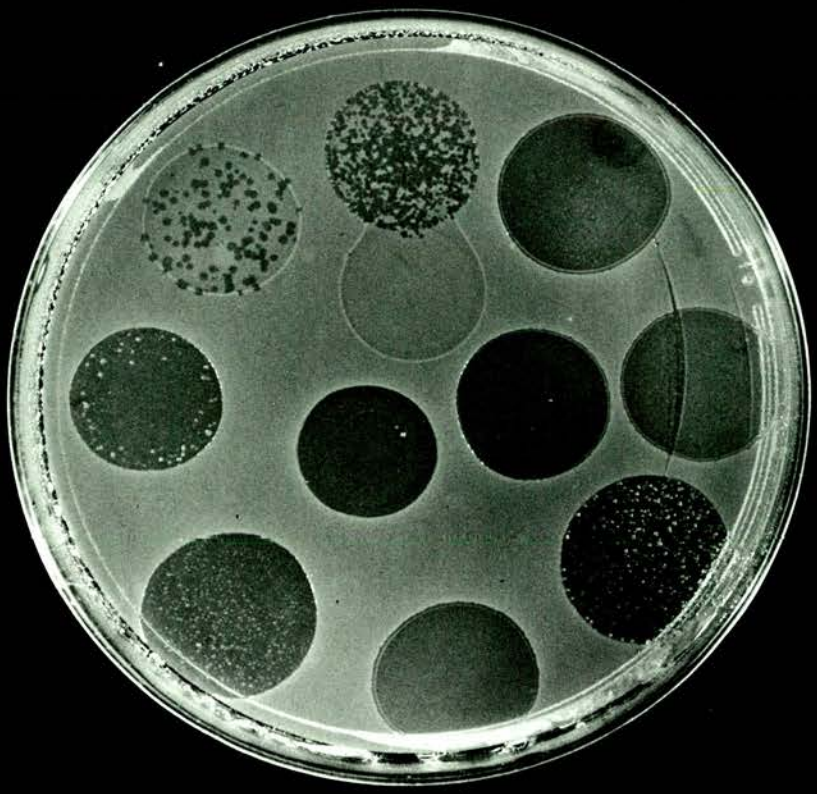
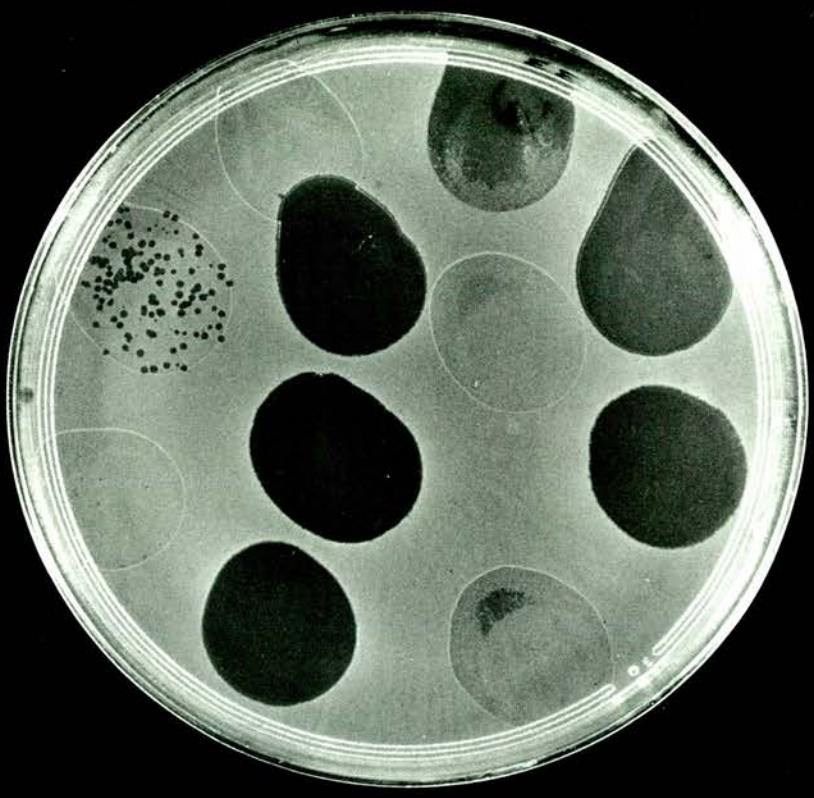


PLATE 68



Plates 67 and 68. Effects of the battery of 11 phages used at R.T.D. on 2 different strains of Ps.pseudomallei.

PLATE 70

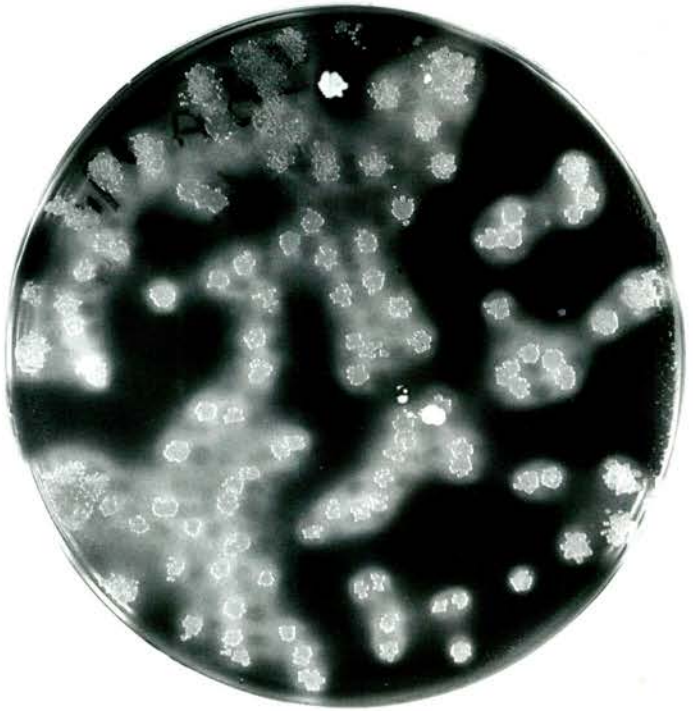


Plate 70. Haemolysis produced when growth (colonies emerging on a plate seeded with Ps.pseudomallei and phage 50/4) was replica-plated to a fresh blood agar plate seeded with phage 50/4.

PLATE 69

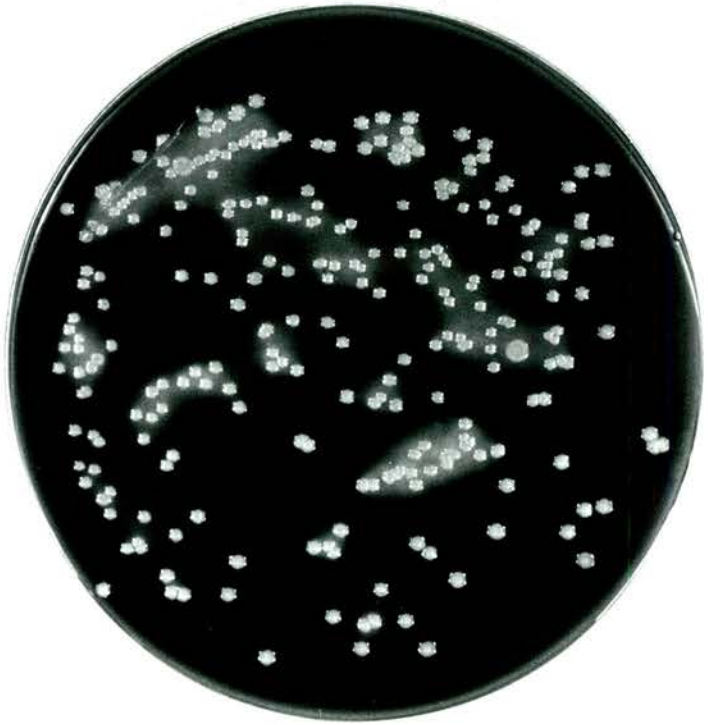


Plate 69. Haemolysis produced within 48 hours on a plate seeded with a mixture of Ps.pseudomallei strain 4-SR and phage 50/4.

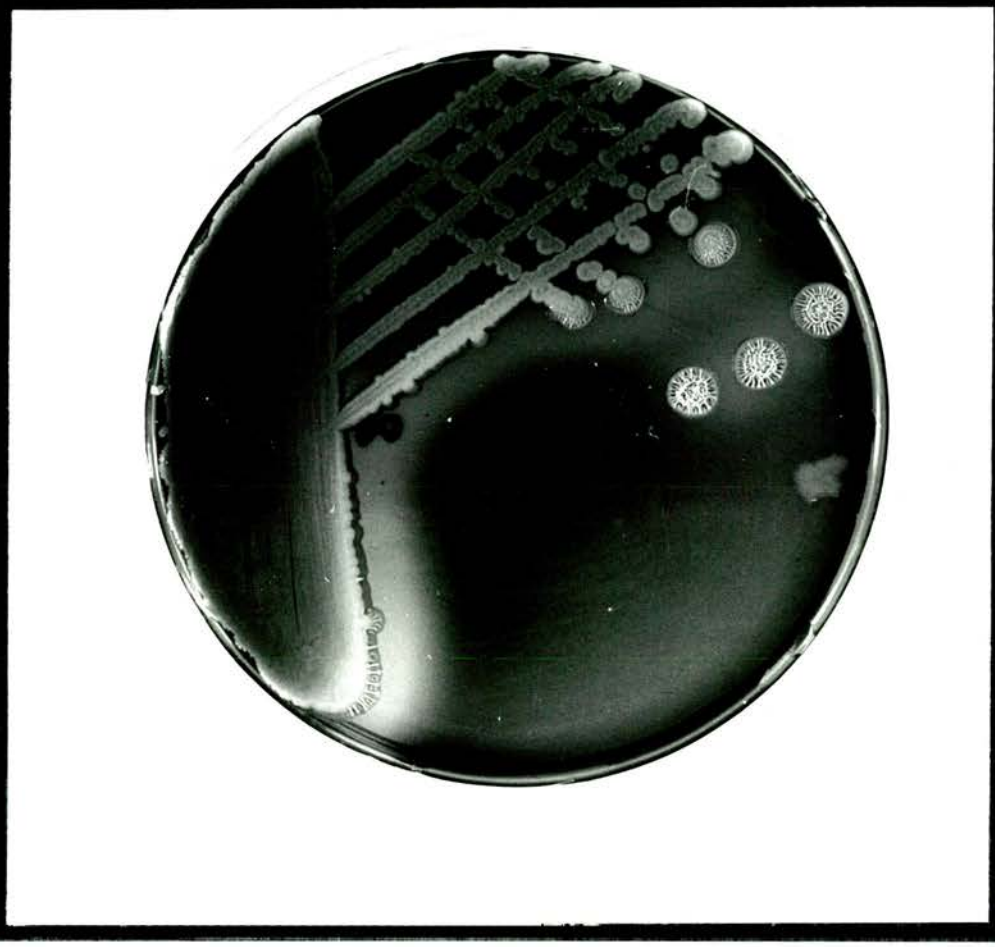


Plate 71. Weak haemolysis produced by Ps.pseudomallei strain PR-4-R.

PLATE 72

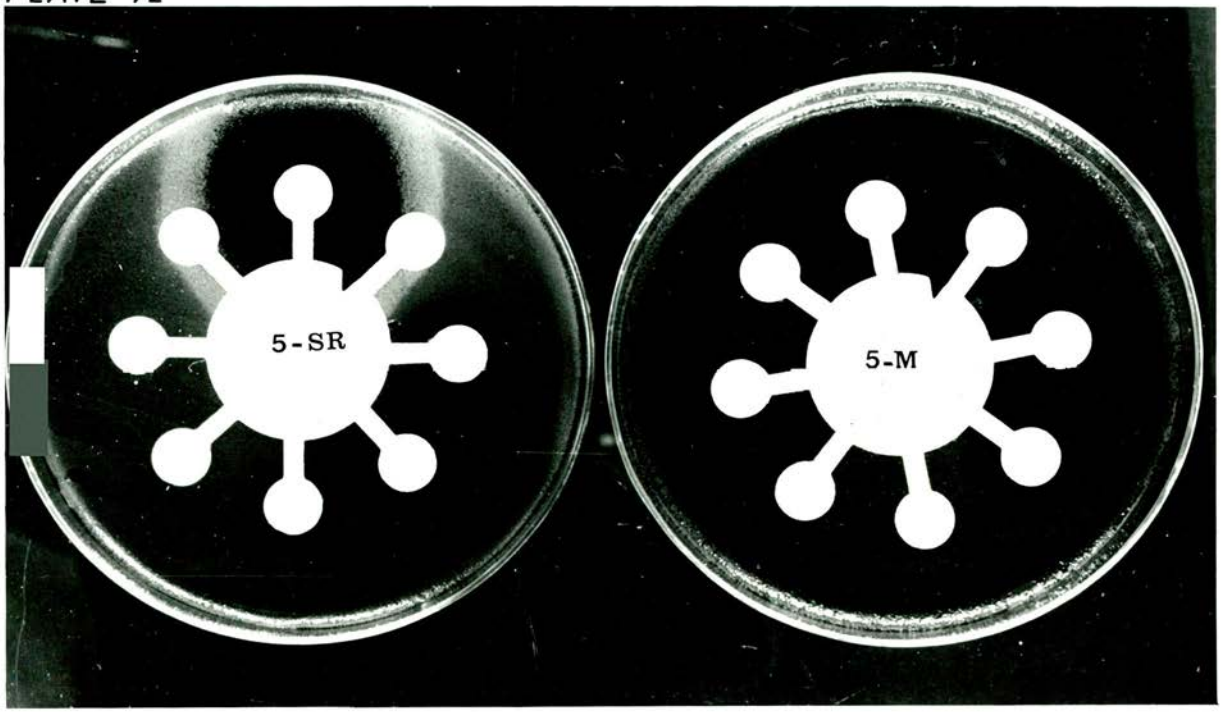


Plate 72. The higher degree of sensitivity to antibiotics shown by the phage-resistant "M" variant (see "b") in contrast to the phage-sensitive parent Ps.pseudomallei - strain 5 (see "a").

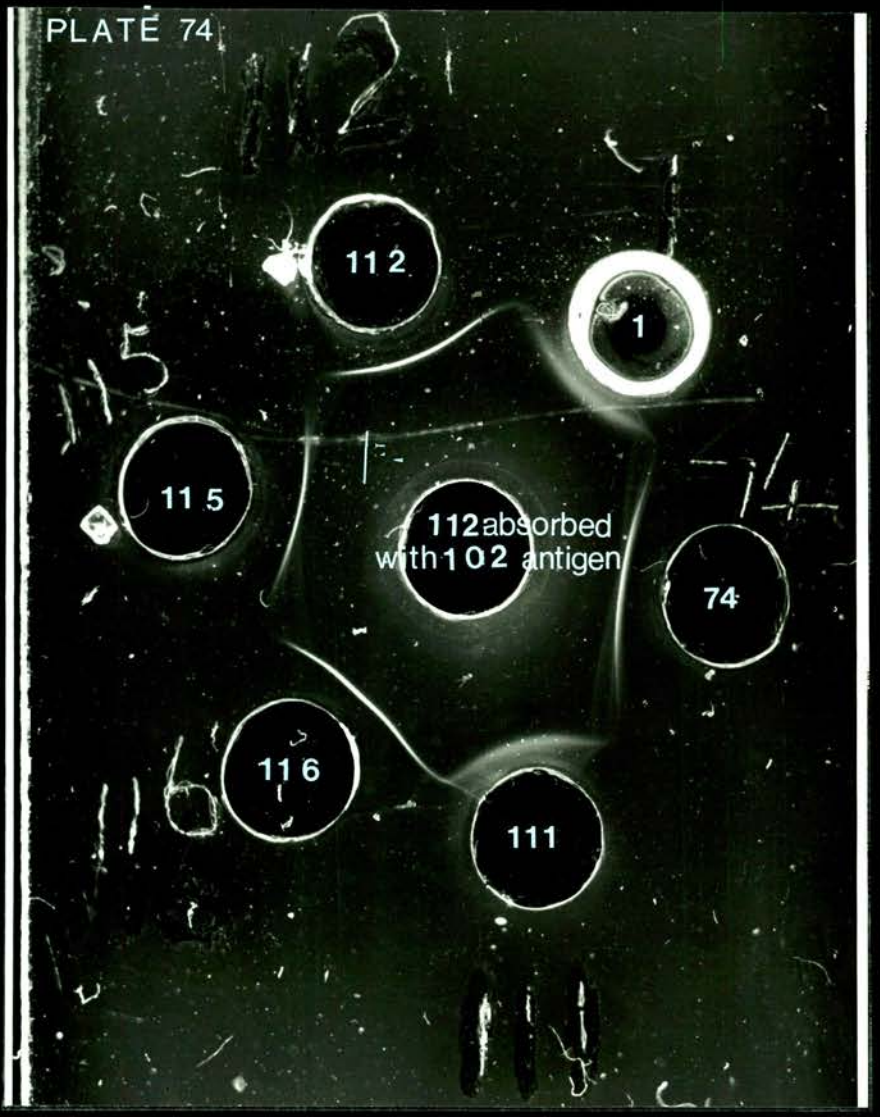


Plate 74. A single precipitin band observed when strains in Group I (112,115, 116,111, 74 & 1) were tested against antiserum - 112 absorbed with antigen 102.

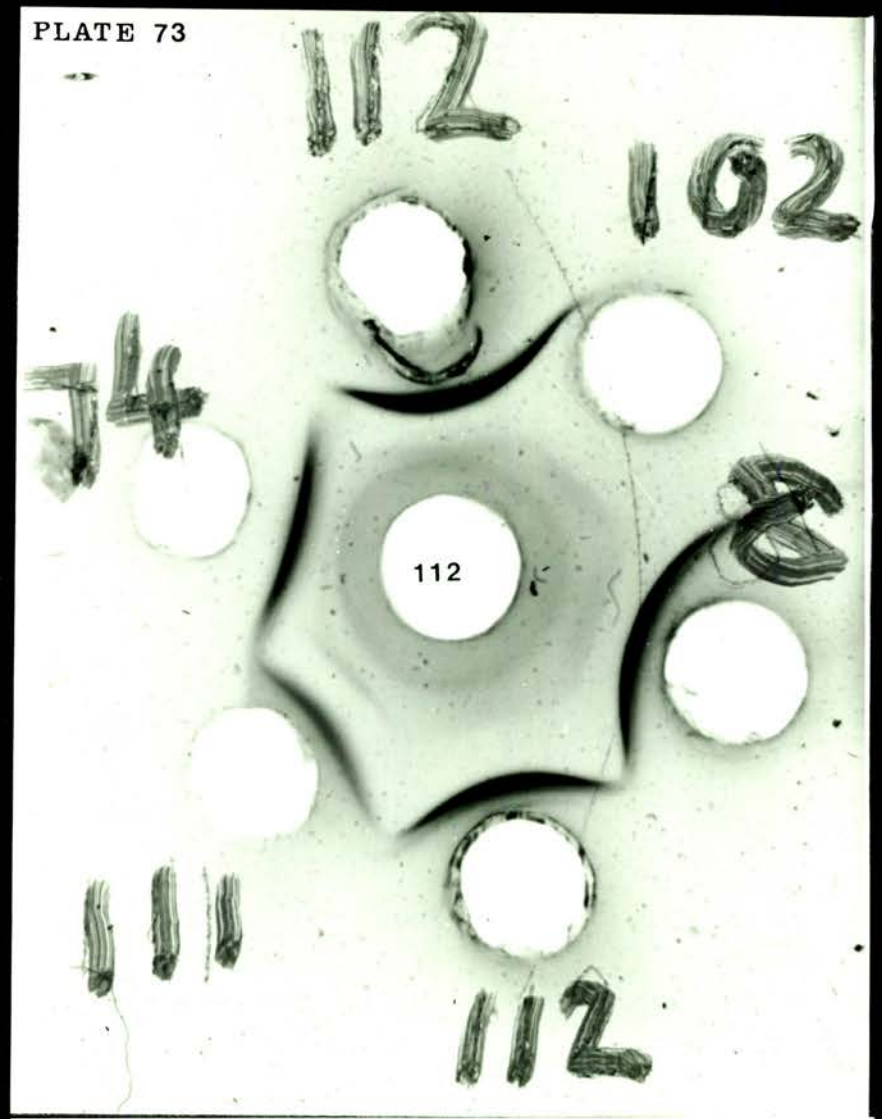


Plate 73. The crude aqueous extract from Ps.pseudomallei strains 112,74, 111 & 8 have reacted with anti-serum - 112 (central well) to produce 2 precipitin bands. The extract from strain 102 has reacted to produce a single band.

PLATE 76

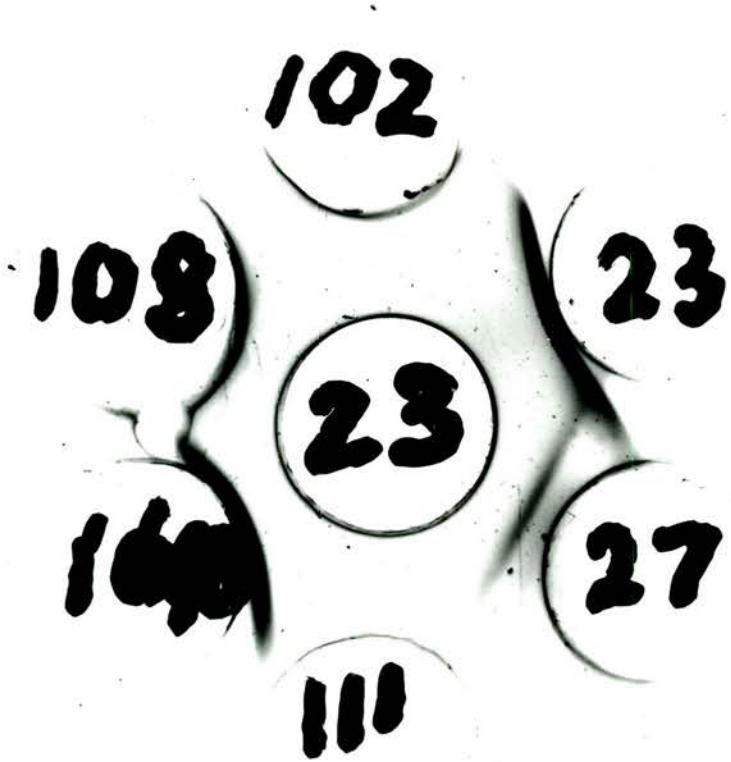


Plate 76. Antiserum-23 absorbed with antigen-102 has retained an antibody fraction or fraction to strains 23,27 and 14. The precipitin band produced by antigen-27 does not show idenity with that of antigen-23.

PLATE 75

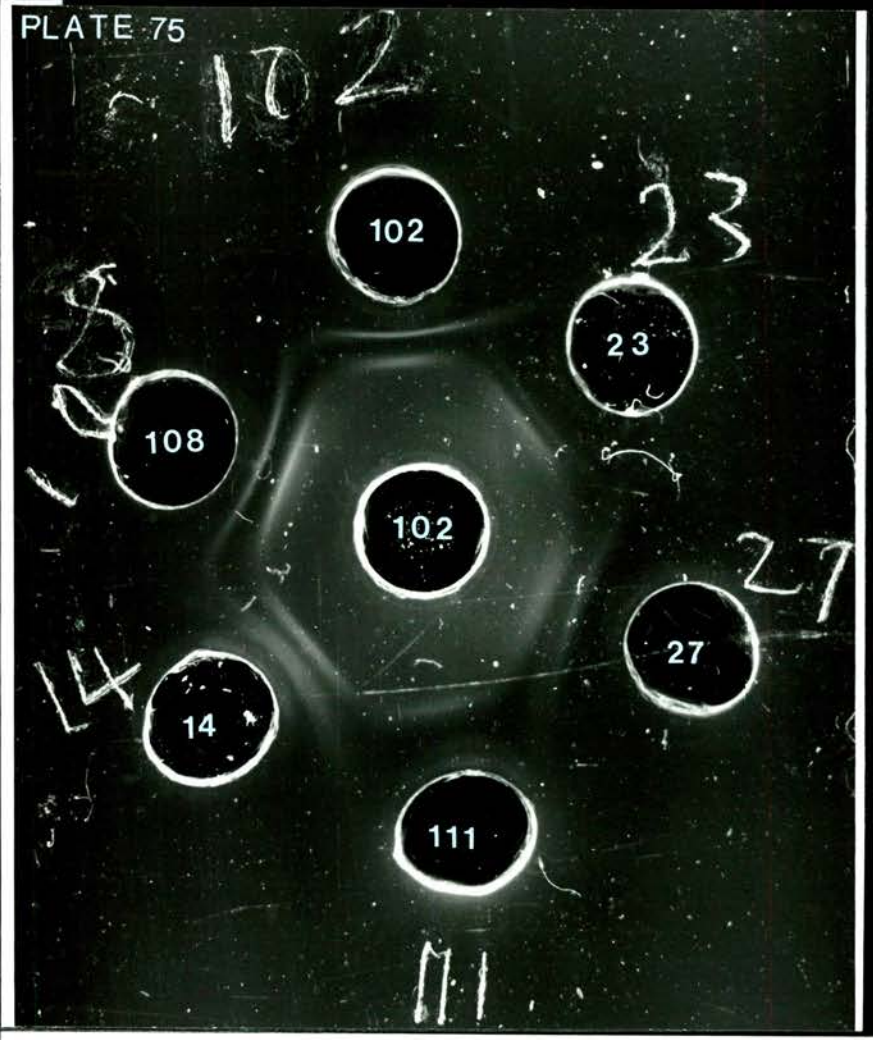


Plate 75. Crude aqueous extract of Ps.pseudo-mallei strains 102,108,14,27 & 23 have produced 2 precipitin bands when tested against antiserum - 102 (central well). Strain 111 belonging to Group I has produced only a single band.

PLATE 78

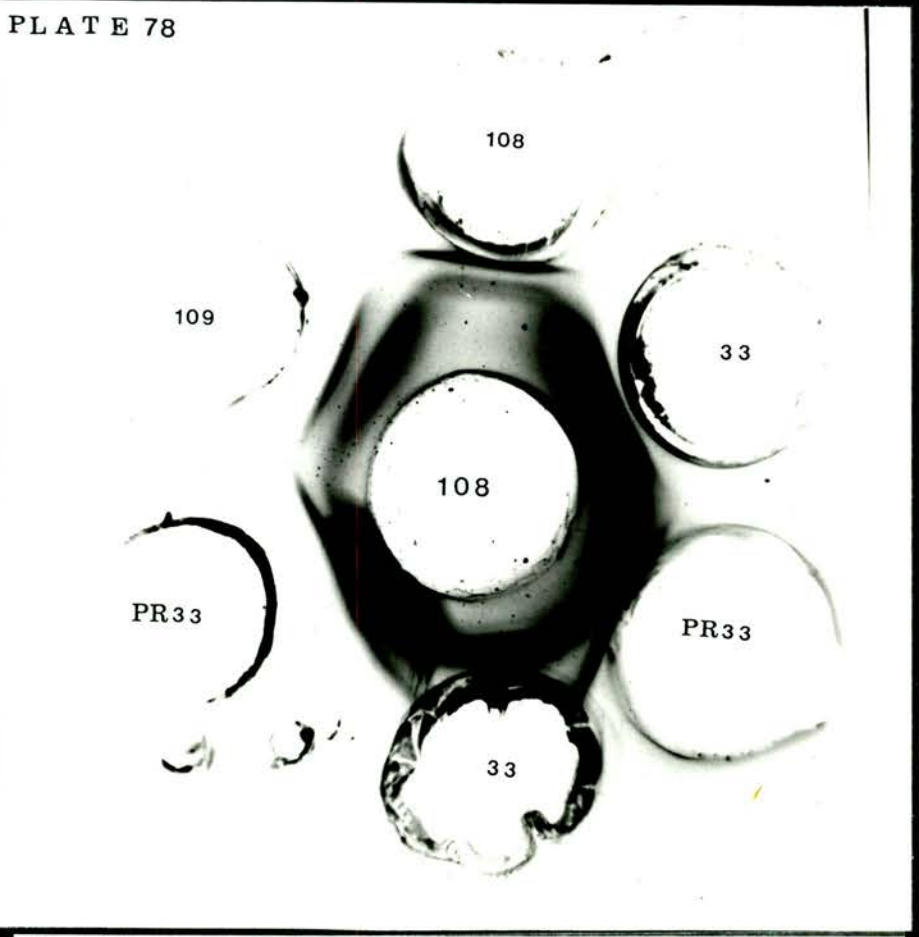


Plate 78. Antigen-PR33 unlike antigen-33 has produced 2 precipitin bands against antiserum-108 (central well)

PLATE 77

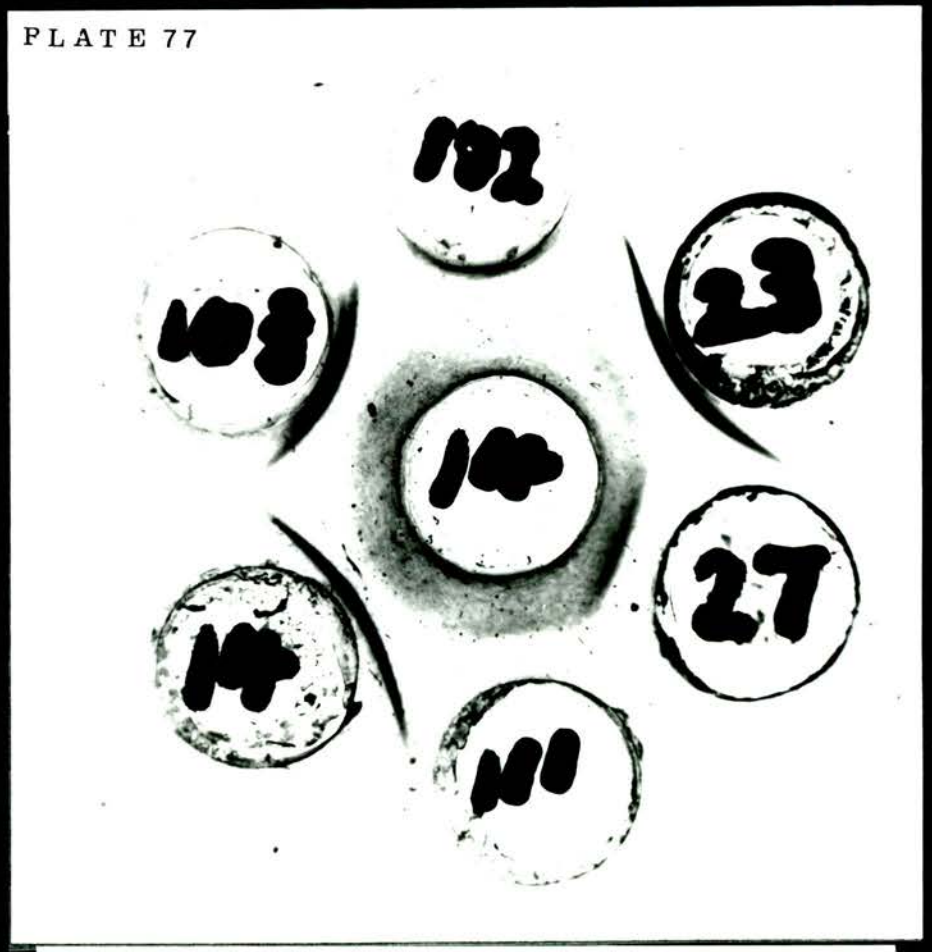


Plate 77. Serum-14 absorbed with antigen-102 has retained an antibody fraction or fractions to strains 14,23,27 and 108.

PLATE 80

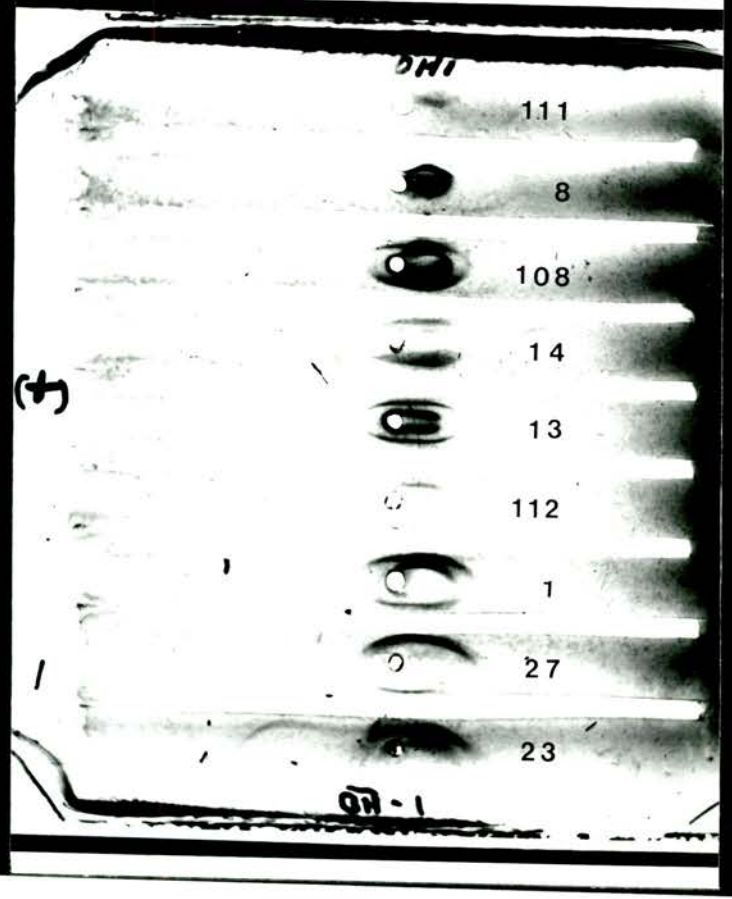


Plate 80. Immuno-electrophoresis. Troughs contain antiserum produced against "heated vaccine" of Ps.pseudomallei strains1. Antigens 108, 14, 13, 27, 23 and 1 have reacted to produce 3 bands.

PLATE 79

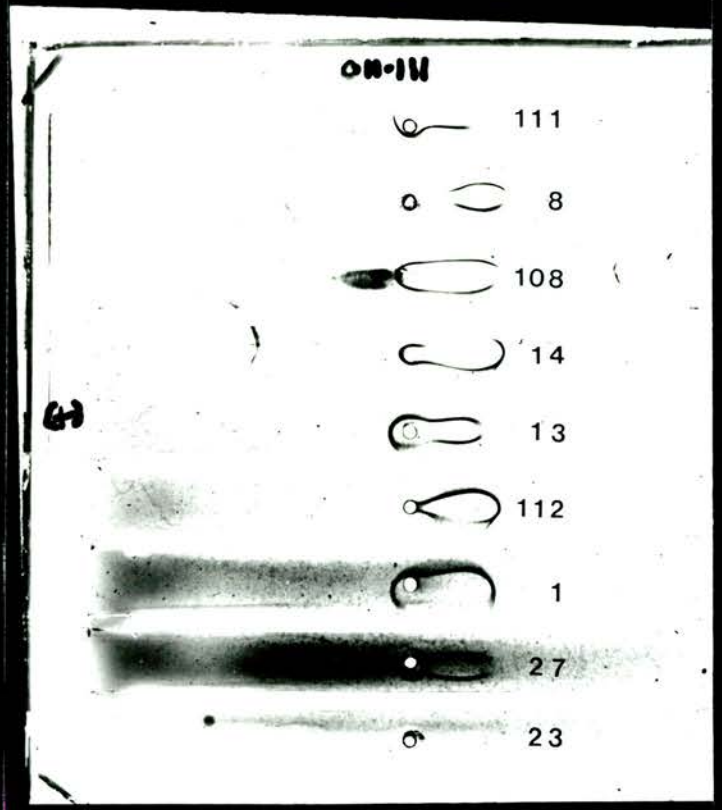


Plate 79. Immuno-electrophoresis. Troughs contain antiserum produced against the "heated vaccine" of Ps.pseudomallei strain 111. The homologous strain has reacted to produce 2 precipitin bands. Others, mostly strains of Group IIa and IIb, have produced a single band.

PLATE 82

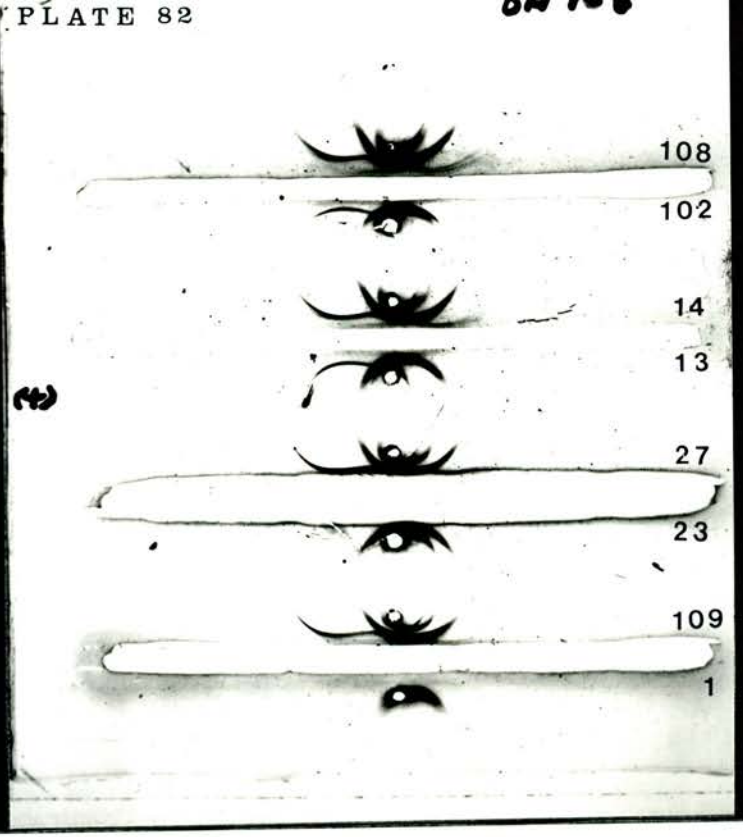


Plate 82. Immuno-electrophoresis. Troughs contain antiserum produced against "heated vaccine" of Ps.pseudomallei strain 108. Up to 6 bands can be seen against antigens 108,102, 13, 14, 27, 23 and 109.

PLATE 81

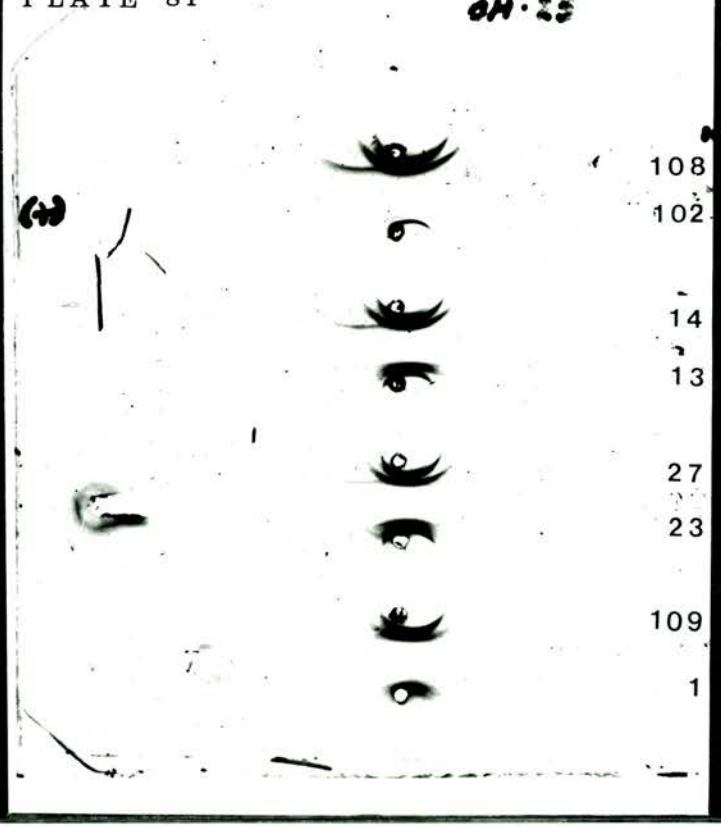


Plate 81. Immuno-electrophoresis. Troughs contain antiserum produced against "heated vaccine" of Ps.pseudomallei strain 23. It has reacted to show 3 precipitin bands against antigen of the homologous strain and against antigens 13, 14, 27, 108 and 109. Antigen of strain 102 has reacted to produce a single band.